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Short Communication

Viviparous-1 Mutation in Maize Conditions Pleiotropic Enzyme Deficiencies in the Aleurone[1]

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ABSTRACT

The *viviparous-1* (*vp1*) mutation in maize (*Zea mays* L.) conditions a unique pleiotropic phenotype: premature germination of the embryo and failure to synthesize anthocyanin (flavonoid) pigments in the aleurone. By using a *B-A* translocation, it is possible to analyze the basis for the anthocyaninless phenotype of *vp1* in the absence of vivipary. Anthocyaninless *vp1* aleurones were found to be deficient in at least three enzymes of flavonoid biosynthesis (phenylalanine ammonia lyase, chalcone synthase, and UDPG-flavonoid glucosyltransferase) as well as in several other metabolically unrelated enzymes that show pronounced increases in late stages of aleurone development. The set of structural genes encoding such enzymes is postulated to be under the regulation of the *vp1* gene.

Vivipary or premature germination in maize is an alteration of normal seed development that can result from mutation at any one of several genes (11). Some viviparous mutants also affect carotenoid biosynthesis in the endosperm and developing seedling. Immature embryos from these mutants (*vp2, vp7, vp9, ws*) have reduced levels of ABA (1, 16, 17) and their growth is arrested when explanted into culture media containing ABA (12, 16). These mutants have been postulated to represent blocks in ABA biosynthesis.

The *vp1*[3] mutation, on the other hand, has a unique pleiotropic effect. Carotenoid biosynthesis is not affected and the ABA content in developing embryos is normal; growth of *vp1* embryos in culture is not inhibited by ABA (16). Instead, anthocyanin biosynthesis in the aleurone layer of the endosperm is blocked by the mutation. The biochemical basis for the viviparous phenotype of the *vp1* mutation is not known. Smith *et al.* (16, 17) and Robichaud *et al.* (12) have suggested that *vp1* is defective in some element of ABA response, *e.g.* in a specific ABA receptor. Wilson *et al.* (20) and Dooner and Nelson (6) have discussed the possibility that *vp1* may interfere with the synthesis of a precursor common to the anthocyanins and phenylpropanoid germination inhibitors such as coumarin or ferulic acid (19).

Anthocyaninless *vp1* endosperms are deficient in CHS, the first enzyme unique to flavonoid biosynthesis (4) and in UFGT, an enzyme catalyzing one of the last steps in anthocyanin biosynthesis (5). Therefore, it became of interest to determine whether earlier enzymes in the pathway leading from the aromatic amino acids to the phenylpropanoid precursors of the flavonoids (7) would be affected by the *vp1* mutation.

The enzyme PAL catalyzes the deamination of phenylalanine to cinnamic acid and is considered a key enzyme since it channels aromatic amino acid metabolism in the direction of flavonoid biosynthesis. The present study began with an examination of the effect of the *vp1* mutation on PAL levels in developing aleurones. This communication reports data showing that the *vp1* mutation not only affects PAL levels, but also those of other enzymes—metabolically unrelated—that are made late in aleurone development.

MATERIALS AND METHODS

Biological Materials. The lines of maize (*Zea mays* L.) used in this study were in the genetic background of the inbred W22 and carried all the complementary factors required for anthocyanin pigmentation in the aleurone. One line was heterozygous for the recessive *vp1* mutation which maps to the long arm of chromosome *3* (*3L*).

To examine the effects of the *vp1* mutation itself in the absence of the viviparous embryo condition regularly associated with the mutation, advantage was taken of the *TB-3La* translocation which makes it possible to generate embryos and endosperms of noncorresponding genotypes (11). *TB-3La* is a translocation between the heterochromatic *B* chromosome and *3L* that uncovers *vp1*. As used in this text, *TB-3La* symbolizes a translocation heterozygote carrying one normal chromosome *3* plus the translocation pair 3^{B} and B^{3}. Since the B^{3} chromosome, which carries the functional allele of *vp1*, fails to disjoin normally at the second pollen mitosis, the genetic constitution of the two sperm nuclei in the pollen is oftentimes not the same. One sperm receives two copies of the normal allele of *vp1* localized in B^{3} and the second one receives none. The products of the double fertilization of the egg and central cell of the embryo sac by such dissimilar sperms have noncorresponding genotypes. Thus, it is possible to obtain from the cross +/*vp1* × *TB-3La* unique kernel classes having the following aleurone-embryo phenotypes: (a) colored aleurone (*vp1 vp1*/++ hyperploid), viviparous embryo (*vp1*/− hypoploid); (b) small seed, colorless aleurone (*vp1 vp1*/− hypoploid), dormant embryo (*vp1*/++ hyperploid); and (c) small seed, colored aleurone (++/− hypoploid), dormant embryo (+/++ hyperploid). The small seed effect of B^{3} hypoploidy in the endosperm can

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[3] Abbreviations: *vp1*, *viviparous*-1; CHS, chalcone synthase; UFGT, UDPG-flavonoid glucosyltransferase; PAL, phenylalanine ammonia lyase; UDPG, uridine-diphosphoglucose; *TB-3La*, a translocation between the heterochromatic *B* chromosome and the long arm of chromosome *3*; G6PDH, glucose-6-phosphate dehydrogenase; ADH, alcohol dehydrogenase; DAP, days after pollination.

first be detected in kernels that are 4 to 5 weeks old. Class 2 kernels permit an examination of the effect of the *vp1* mutation in the aleurone in the absence of vivipary. Furthermore, class 2 and class 3 aleurones have identical chromosome constitution and differ only in their *vp1* genotype.

Enzyme and Protein Assays. UFGT was assayed as described (3, 6). Total protein was assayed by the method of Potty (10). UDPG pyrophosphorylase was measured following the procedure of Dickinson and Preiss (2) as modified by Hannah and Nelson (8) with the difference that UTP replaced ATP in the reaction mixture. One unit of enzyme corresponds to the formation of 1 nmol UDPG/min. Sucrose synthase was measured in the direction of sucrose synthesis by the method of Tsai *et al.* (18), except that 0.1 M Na borate (pH 8.7) replaced Hepes in the reaction mixture.

G6PDH activity was assayed in 0.08 M Tris-HCl (pH 8.2) containing 3 mM $MgCl_2$, 5 mM glucose-6-P, 0.5 mM NADP, and enzyme in a final volume of 1 ml. The formation of NADPH was measured spectrophotometrically by following the increase in A_{340}, which was found to be linear with time over a period of 30 min. One unit of enzyme as defined in Figure 1 stands for a change of 1 absorbance unit/min. Catalase activity was assayed in 0.05 M K-phosphate (pH 7.3) containing 0.06 M H_2O_2, and enzyme in a volume of 1 ml. The disappearance of peroxide over a period of 2 min was followed spectrophotometrically at 240 nm. ADH was assayed in 0.1 M Na borate (pH 8.7) containing 0.4 M ethanol. Two mM NAD and enzyme in a volume of 1 ml. The reaction, measured as the rate of increase in A_{340}, was linear over a period of 30 min.

The PAL reaction mixture contained 15 nmol [U-^{14}C]phenylalanine (2 Ci/mol), 5 μmol Na borate buffer (pH 8.7), and enzyme in a volume of 50 μl. Tubes were incubated at 37° C for 0 to 30 min. The reaction was stopped by addition of 25 μl ethanol; a 25 μl aliquot of the reaction was spotted on a silica gel thin layer plate (E. Merck) and the chromatogram was developed in a toluene:ethyl formate:formic acid (10:9:1) solvent system. Cinnamic acid standards were run on all chromatograms. The cinammic acid spot was identified under UV light, cut out, and counted in a liquid scintillation counter. One unit of activity corresponds to 1 μmol cinnamic acid synthesized/h.

Enzyme Preparation. Immature seeds harvested at various postpollination times were frozen in dry ice and stored at −20° C until processing. In preparation for extraction, the pericarp was removed from each seed and the aleurone layer was peeled off or scraped from the underlying starchy endosperm. For each developmental time-point and each genotype in Figure 1, 25

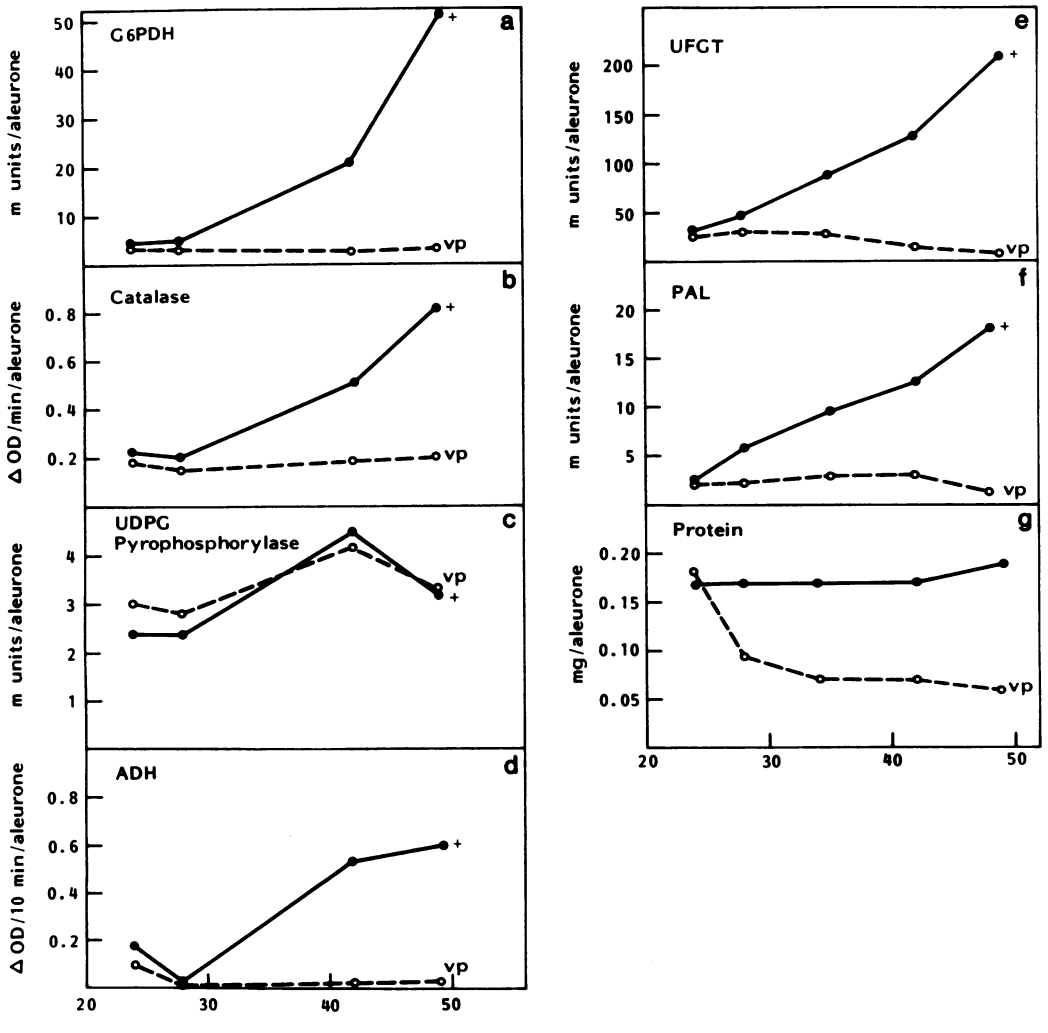

FIG. 1. Developmental profiles of several enzymes and total protein in + (●) and vp (○) aleurones obtained from the kernel classes 2 (vp) and 3 (+) described in "Materials and Methods." a, G6PDH; b, catalase; c, UDPglucose pyrophosphorylase; d, ADH; e, UFGT; f, PAL; g, water-soluble protein.

aleurones were homogenized in a ground glass homogenizer with 3 ml 0.1 M Na borate (pH 8.7) buffer. The crude homogenate was stirred for 15 min with 1 g of AG1-X2 resin previously equilibrated in the extraction buffer and centrifuged at 27,000*g*. The supernatant was dialyzed against either extraction buffer or 0.1 M Tris-HCl (pH 8.2) and used in enzyme assays.

RESULTS AND DISCUSSION

The effects of the *vp1* mutation itself on the levels of various enzymes localized in the aleurone layer of the maize seed can be separated from those caused by the premature germination of the embryo by examining noncorresponding classes of kernels arising from the cross +/*vp1* × *TB-3La* (see "Materials and Methods"). Aleurones from the small dormant kernels obtained from this cross are either colorless or colored: the colorless aleurones are *vp1 vp1*/− hypoploids, whereas the colored ones are ++/− hypoploids. These are designated vp and +, respectively, in Figure 1.

Developmental curves for the activities of several enzymes in the aleurone are presented in Figure 1. The + and vp curves for aleurone UFGT (Fig. 1e) are much like those already published for whole endosperm UFGT (6), confirming that the bulk of this flavonoid biosynthetic enzyme is localized in the aleurone and that the *vp1* mutation drastically affects its levels.

Since a premise under consideration was that the *vp1* pleiotropic effect could result from a block in phenylpropanoid metabolism, levels of the first enzyme of phenylpropanoid biosynthesis (PAL) were compared in + and *vp1* aleurones. Early in development (24 DAP), PAL activity is low in both genotypes. However, whereas *vp1* aleurones retain this low, residual enzyme level throughout development, normal aleurones show a continuous increase in PAL activity up to 49 DAP (Fig. 1f). The reduced levels of PAL should result in a deficiency in phenylpropanoid derivatives in tissues where the *vp1* gene is known to be expressed. If a compound of this nature does act as a dormancy factor in maize, the premature germination condition seen in *vp1* embryos would be explained.

An unexpected observation made during the course of these studies was that in *vp1* aleurones, but not in + aleurones, there is a progressive decrease in the amount of total water-soluble protein from 24 to 49 DAP (Fig. 1g). Protein measurements done on whole endosperms generated the same way (*i.e.*, from +/*vp1* × *TB-3La* crosses) had not revealed this difference (6). Clearly, *vp1* aleurones could be deficient in other enzymes, unrelated to flavonoid metabolism, that appear to be synthesized late in the development of the aleurone. Though developmental profiles for enzymes that appear in the endosperm during the first 4 weeks after pollination—such as starch biosynthetic enzymes—have been described (18), enzymes made late in development or specifically in the aleurone tissue have not been examined in much detail. In an attempt to define the developmental profile of such enzymes, the levels of several enzymes were measured: G6PDH, catalase, ADH, UDPG pyrophosphorylase, and sucrose synthase. As seen in Figure 1, a, b, and d, levels of the first three enzymes rose markedly between 24 and 49 DAP. That is, G6PDH, catalase, and ADH can be classified as 'late' enzymes in aleurone development. All of these are affected by the *vp1* mutation in a manner analogous to PAL and UFGT. On the other hand, UDPG pyrophosphorylase levels, which did not increase appreciably between 24 and 49 DAP, were not affected by the *vp1* mutation. Finally, sucrose synthase could not be detected in normal or *vp1* aleurones at any stage of development (data not shown). This was not surprising in view of the facts that this enzyme functions in starch synthesis and that the aleurone, in contrast to the rest of the endosperm, accumulates little starch.

The results presented here show that the *vp1* mutation has a broad pleiotropic effect. Proteins synthesized late in development do not appear to be made in *vp1* aleurones: the levels of all the enzymes examined that showed pronounced increases late in development were affected by *vp1*. Except for ADH, these enzymes also have been reported to be under phytochrome control in higher plants (14), although the significance of this observation can only remain conjectural at this time. The structural genes for several of these endosperm enzymes are known and are scattered in the maize genome: UFGT-*bz* (9, 5); CHS-*c2* (4); ADH-*Adh1* (15); catalase-*Cat1* and *Cat2* (13). Thus, *vp1* may be a major regulatory gene responsible for 'turning on' a battery of structural genes at a certain time in development. The *vp1* gene is highly tissue specific, being functional only in the aleurone and the developing embryo. Evidently, it is not active at later stages of development because *vp1* seedlings are not impaired in their capacity to synthesize anthocyanins and develop eventually into normal—though somewhat shorter—plants.

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