INTERACTION AMONG *C*, *R* AND *Vp* IN THE CONTROL OF THE *Bz* GLUCOSYLTRANSFERASE DURING ENDOSPERM DEVELOPMENT IN MAIZE[1]

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ABSTRACT

The enzyme UPD glucose: flavonoid 3-*O*-glucosyltransferase (UFGT), involved in anthocyanin biosynthesis, is controlled by at least four genes (*Bz*, *C*, *R* and *Vp*) in the maize endosperm. *Bz* is the structural gene for the enzyme. Early in endosperm development, the enzyme is present in an uninduced low level that is independent of *C*, *R* and *Vp*, and dependent solely on *Bz*. Beginning at about the fourth week of development, *C*, *R* and *Vp* interact with each other to induce high levels of UFGT. The enzyme accumulates thereafter in normal endosperms, reaching its highest level at maturity. The nature of the developmental signal (s) controlled by *C*, *R* and *Vp* is discussed.

THE enzyme UDP glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) catalyzes one of the terminal steps in anthocyanin biosynthesis, namely, the 3-*O*-glucosylation of anthocyanidins and the related flavonols. In maize endosperms this enzyme has been shown to be under the coordinate control of at least three genes, scattered in the genome: *Bz* (*9S*), *C* (*9S*, six units distal to *Bz*) and *R* (*10L*). Stable mutants in the *Bz* locus, conditioning bronze aleurone color, are null, totally lacking UFGT activity. Furthermore, in triploid endosperms, the *Bz* normal allele has a clear linear dosage effect on activity. It appears that *Bz* is the structural gene for UFGT (LARSON and COE 1977; DOONER and NELSON 1977a). The *c* and *r* mutants are colorless aleurone mutants that display low (2–3%) levels of UFGT activity in mature endosperms. However, variation of *C* and *R* dosage against the respective recessives does not significantly affect UFGT levels (DOONER and NELSON 1977a). Because of: (1) the gene dosage-enzyme relationships described above, and (2) accumulating evidence indicating that *C* and *R* act very early in the anthocyanin biosynthetic pathway (*e.g.*, REDDY and COE 1962; KIRBY and STYLES 1970; STYLES and CESKA 1977; MCCORMICK 1978), it would seem that *C* and *R* regulate, in an as yet undetermined way, UFGT levels in the endosperm.

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Evidence is reported in this paper that mutation in still another gene, *Vp* (located in *3L*), reduces UFGT activity in mature endosperms to the same extent as do mutations in *C* and *R*. Developmental profiles of UFGT activity in normal, *vp*, *c*, *r*, and *bz* endosperms are presented, showing that early in development the first four genotypes listed have roughly the same residual enzyme level, whereas *bz* endosperms totally lack activity. Later, however, *c*, *r* and *vp* endosperms apparently lack a developmental signal that, in normal endosperms, triggers the *Bz* locus to produce increasingly higher levels of UFGT beginning at about the fourth week of development and continuing through to maturity. The significance of this apparently complex control of UFGT activity is discussed.

MATERIALS AND METHODS

Stocks: The lines used in the present study were in the common genetic background of the inbred W22. These lines carry all the complementary factors required for purple anthocyanin pigmentation in the aleurone, with the exception of one of the following recessives:

bz-R (*Bz* locus reference allele, chromosome *9*): bronze aleurone.

c-p (*C* locus allele, chromosome *9*): colorless aleurone.

r-g (*R* locus allele, chromosome *10*): colorless aleurone.

vp-R (*Vp* locus reference allele, chromosome *3*): colorless aleurone, viviparous embryo. Propagated in heterozygous condition, although the homozygote is viable if rescued before maturity.

TB-3La: a translocation between the heterochromatic *B* chromosome and the long arm of chromosome *3* (*3L* 0.10) that uncovers *vp*. Propagated as a heterozygote: therefore, as used in this paper, *TB-3La* symbolizes a translocation heterozygote carrying one normal chromosome *3* plus the translocation pair 3^{B} and B^{s}.

+/vp × TB-3La *cross:* Since the B^{s} translocation chromosome undergoes nondisjunction at the second male gametophytic mitosis, it is possible to generate the following unique kernels from the cross +/*vp* × *TB-3La* (ROBERTSON 1955): (1) small, colored endosperm (++/— hypoploid), dormant embryo (+/++ hyperploid); (2) small colorless endosperm (*vp vp*/— hypoploid), dormant embryo (*vp vp*/++ hyperploid), and (3) colored endosperm (*vp vp*/++ hyperploid), viviparous embryo (*vp*/— hypoploid). The small seed effect of B^{s} hypoploidy in the endosperm can be detected in kernels that are 28 days old or older. Class 2 kernels permit an examination of the effect of the *vp* mutation in the endosperm when a dormant embryo is present, *i.e.*, in the absence of the premature germination condition seen in *vp* homozygotes. Furthermore, class 1 and class 2 endosperms have identical chromosome constitution and differ only in their *vp* locus genotypes.

Enzyme and protein assays: UFGT activity and total protein were assayed as described by DOONER and NELSON (1977a). The following modifications were introduced. Incubation times of 0, 15 and 20 min were used for normal enzyme preparations obtained from early developmental stages and for enzyme preparations from the mutants obtained throughout development, The times were shortened to zero, five and ten min when enzyme was extracted from colored kernels harvested beyond 28 days after pollination. Labeled isoquercitrin, the product of the reaction, was routinely separated from the substrates, UDP glucose and quercein, by paper chromatography (Whatman 1 paper developed in 15% acetic acid). A unit of enzyme is defined as that producing 1 μm of isoquercitrin per hour under the standard assay conditions.

Enzyme preparation: The preparation of UFGT from mature endosperms and frozen, immature endosperms collected at various times after pollination has been described (DOONER and NELSON 1977a,b). In all instances, the crude enzyme preparation from endosperms manually separated from their embryos was treated with an ion-exchange resin and concentrated by precipitation with ammonium sulfate (30–55% saturation). The precipitate was resuspended

in 1/10 the initial volume of buffer and dialyzed overnight against the same buffer. The dialysate was then used in enzyme assays.

RESULTS

UFGT activity in + and vp *mature endosperms*: Enzyme levels in mature colored (+) and colorless (*vp*) seed segregants from both *vp*/+ × *vp*/+ and *vp*/+ × *TB-3La* (see MATERIALS AND METHODS) are reported in Table 1. As can be seen from the data, *vp* endosperms have very low UFGT levels at maturity. Since the *vp* mutation also conditions premature embryo germination, this abnormal physiological condition could account for the reduced enzyme activity seen in *vp* kernels from *vp*/+ × *vp*/+ matings. However, the colorless *vp vp*/— hypoploid endosperms obtained from the cross *vp*/+ × *TB-3La*, which are associated with a dormant embryo, also have a low activity level when compared with their colored ++/— hypoploid sib controls. These data argue that the low UFGT activity seen in *vp* mature endosperms is a direct effect of the mutation and not of the pleiotropic viviparous embryo condition regularly associated with the mutation.

That vivipary *per se* does not lead to a reduction in UFGT activity as great as that seen in *vp* endosperms is shown by the activity level of class 3 kernels from the cross *vp*/+ × *TB-3La*. These kernels have a colored endosperm (*vp vp*/++) and a viviparous embryo (*vp*/—). They result from the reciprocal fertilization event that gives rise to class 2 kernels. Enzyme activity in these kernels is reduced to about one-half the normal level. Whether this reduction is due to premature germination of the embryo or to the fact that the two doses of the normal allele are male- rather than female-contributed (*cf.* KERMICLE 1970) cannot be ascertained at the present time.

UFGT activity in +, vp, c, r *and* bz *endosperms during development*: Figure 1 shows UFGT levels measured at various post-pollination times in frozen, im-

TABLE 1

UFGT levels in dry, mature endosperms obtained from the matings: (*A*) vp/+ × vp/+; *and* (*B*) vp/+ × TB–3La (*see* MATERIALS AND METHODS)

Mating	Kernel class	Phenotype		Genotype		Endosperm designation	Specific activity*	Percent normal†
		Endosperm	Embryo	Endosperm	Embryo			
A	1	colored	dormant	++/+ ++/*vp* *vp vp*/+	+/+ +/*vp* *vp*/+	+	224	100
	2	colorless	viviparous	*vp vp*/*vp*	*vp*/*vp*	*vp*	0.9	0.4
B	1	colored, small	dormant	++/—	+/++	+	304	100
	2	colorless, small	dormant	*vp vp*/—	*vp*/++	*vp*	7	2.3
	3	colored	viviparous	*vp vp*/++	*vp*/—		149	49

* Enzyme milliunits/endosperm.
† Similar percentages are obtained if specific activity is expressed on a per mg protein basis.

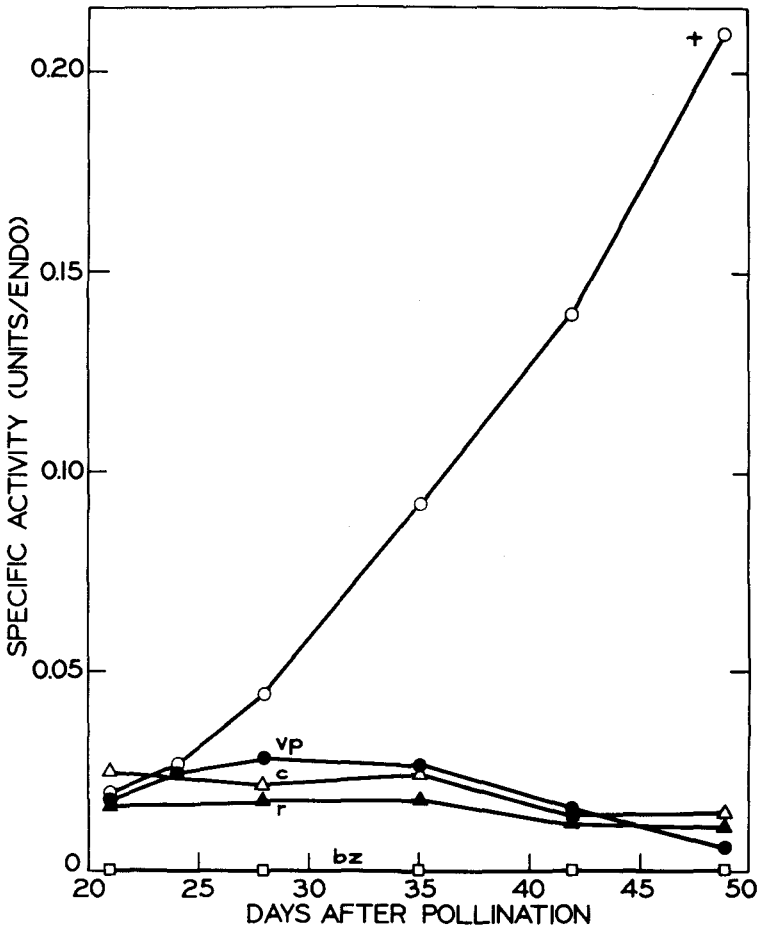

FIGURE 1.—UFGT activity during endosperm development in the + (○——○) and *vp* (●——●) types resulting from the cross *vp*/+ × *TB-3La* (see Table 1), and in *c-p* (△——△), *r-g* (▲——▲) and *bz-R* (□——□) homozygotes.

mature endosperms of the following types: + and *vp*, obtained from the cross *vp*/+ × *TB-3La* (see Table 1), and *c*, *r* and *bz*, obtained from the respective homozygotes. The main points illustrated in Figure 1 are: (a) All genotypes, except *bz*, have roughly comparable levels of UFGT activity very early in development (21 days after pollination). (b) Activity in normal (+) endosperms rises continuously during development and actually reaches a maximum at physiological maturity. A similar developmental curve for normal endosperms extracted from homozygotes has already been described (DOONER and NELSON 1977b). (c) Enzyme activity in *c*, *r* and *vp* endosperms remains at a basically low level through the fifth week of development and declines somewhat between the fifth and seventh week. (d) *bz* endosperms lack detectable activity at all developmental stages.

DISCUSSION

The genetic control of anthocyanin pigmentation in the aleurone layer of the maize kernel has long been regarded as a textbook example of genic interaction. The interplay of many genes leads to the formation of the final product. Mutation in at least eight of these genes (*A*, *A2*, *Bz*, *Bz2*, *C*, *C2*, *R* and *Vp*) results in failure of the aleurone to produce anthocyanin pigment.

Current evidence indicates that *Bz* is the structural gene for the enzyme UDP glucose: flavonoid 3-*O*-glucosyltransferase (UFGT), which catalyzes a late step in anthocyanin biosynthesis (LARSON and COE 1977; DOONER and NELSON 1977a). In addition, mutations at *C* and *R*, but not at *A*, *A2*, *C2*, or *Bz2*, drastically reduce UFGT levels at maturity (DOONER and NELSON 1977a). Evidence has been presented in this report that the *vp* mutation also causes a similar reduction in UFGT activity in mature endosperms.

The dry mature kernel constitutes the final stage of endosperm development. To examine the effect of the *c*, *r*, *vp* and *bz* mutations on UFGT activity during kernel maturation, enzyme levels were measured in immature endosperms at several stages of development. The results presented in Figure 1 show that the low UFGT level seen in normal endosperms early in development is not affected by mutation at either *C*, *R* or *Vp*. However, in contrast to the normal genotype, mutants in these loci retain low, residual enzyme levels throughout development. Mutation at *Bz*, the structural gene for UFGT, results in elimination of the enzyme at all developmental stages. These observation are interpreted to mean that the low UFGT level seen early in development, which is independent of *C*, *R* and *Vp* control and solely dependent on the presence of *Bz*, represents an uninduced condition of the *Bz* locus. Later in development, in response to a signal (or signals) generated by the action of *C*, *R* and *Vp*, *Bz* is induced to produce higher levels of UFGT. The enzyme then accumulates in the developing normal endosperms, reaching a maximum at maturity.

The nature of the signal(s) controlled by *C*, *R* and *Vp*, which is (are) responsible for induction of the *Bz* enzyme, remains conjectural. Available evidence based on an inter-tissue complementation technique utilizing pairs of mutants (REDDY and COE 1962), analysis of accumulated intermediates in single and double mutants (KIRBY and STYLES 1970; REDDY and REDDY 1971; STYLES and CESKA 1972, 1977), and response of mutant genotypes to exogenously supplied precursors (MCCORMICK 1978) ,when taken together, indicates that *C* and *R* act prior to *C2*, *A*, *A2*, *Bz* and *Bz2* in the anthocyanin biosynthetic pathway. Mutants in the latter four genes accumulate some type of C_{15} flavonoid intermediate, whereas *c2* is the only known mutant that pigments in response to flavanone, the first C_{15} compound in the pathway. These observations suggest that none of these mutants is deficient in the elaboration of the C_6-C_3 phenylpropanoid moiety (see GRISEBACH and HAHLBROCK 1974; HAHLBROCK and GRISEBACH 1975).

In a previous communication (DOONER and NELSON 1977a), we considered two possible models to explain the interaction between *Bz*, *C* and *R*. According

to the first model, *C* and/or *R* would be regulatory genes coding for macromolecules directly involved in turning on the *Bz* structural gene. Such a macromolecule could even be an early enzyme in the biosynthetic pathway, as is, for example, threonine deaminase in yeast, which appears to act as a positive regulator of isoleucine-valine biosynthetic enzymes (BOLLON and MAGEE 1971; BOLLON 1974). According to the second model, *C* and *R* would specify early enzymes in the anthocyanin pathway involved in the synthesis of a flavonoid precursor responsible for UFGT induction. This precursor induction would most likely be mediated *via* an activated regulatory protein, in a manner analogous to the interaction between α-isopropylmalate and the *leu-3*$^+$ product in inducing leucine biosynthetic enzymes in Neurospora (POLACCO and GROSS 1973).

Since *vp* is the only colorless aleurone mutant listed above that has a pleiotropic effect (premature germination), it was argued that *Vp* could precede *C* and *R* in the anthocyanin biosynthetic scheme. If UFGT is precursor-inducible, and the action of *Vp* precedes those of *C* and *R*, *vp* endosperms should have low UFGT levels, comparable to *c* and *r* endosperms. This has been shown to be so in the present study. Thus, it would appear that UFGT is indeed a precursor-inducible enzyme. However, recent evidence (DOONER and NELSON, unpublished) indicates that *vp* aleurones, generated as described earlier from *vp*/+ × *TB-3La* crosses, are not only deficient in UFGT, but also in the related enzyme phenylalanine ammonia lyase (PAL), the first enzyme in the pathway from aromatic amino acids to anthocyanin, as well as in unrelated enzymes, such as alcohol dehydrogenase. Therefore, the effect of *Vp* on the pathway could be either through direct control of several genes in the pathway (including *Bz*), or, by affecting PAL, through the indirect control of the pool size of cinnamic acid, the flavonoid precursor produced by the action of PAL.

Thus, no clear choice between the two models can be made at this point. It would seem unduly elaborate for all three genes, *C*, *R* and *Vp*, to be involved in the production of macromolecular activators that regulate *Bz* activity. If this were so, mutations in genes specifying the enzymes that metabolize the phenylpropanoid moiety of anthocyanins remain yet to be identified. More likely, the interaction of a flavonoid precursor and a regulatory macromolecule is required for UFGT induction during endosperm development.

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