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EXPRESSION OF THE DEVELOPMENTAL MUTANT *bz-m4 DERIVATIVE 6856* IN MAIZE SEEDLINGS

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Abstract—The *bronze* (*bz*), locus encodes a flavanoid glucosyltransferase (UFGT) which is required for anthocyanin biosynthesis. Enzyme accumulation in the primary root and coleoptile were compared for the wild-type *Bz-McC* allele and the regulatory mutant *bz-m4 Derivative 6856*. UFGT levels are quantitatively reduced in *bz-m4* plant tissues as compared to that of *Bz-McC*; however both alleles had similar developmental profiles.

INTRODUCTION

Mutations affecting expression rather than the structure of a gene product are relatively rare in higher plants. One *Ds* transposable element mutation at the *bz* locus behaves as a regulatory mutant; this is the allele *bz-m4 Derivative 6856* [1]. The mutant seed are deep bronze in colour, with considerably less pigment than those with the wild-type *Bz* allele but noticeably darker than seeds of the null allele, *bz-R*. The gene product, UDPglucose:flavonol 3-*O*-glucosyltransferase (UFGT; EC 2.4.1.91), converts anthocyanidins (the aglycones) to their corresponding anthocyanin [2, 3]. The phenotype of *bz-m4 Derivative 6856* seed suggests that they possess some cyanidin-glycosylating activity [4, 5]. Our long-range goal is to understand how this *Ds* mutation controls gene expression at the molecular level.

In developing seeds, the phenotype of *bz-m4 Derivative 6856* results from an alteration in the temporal pattern of gene expression [4, 5]. In the mutant seeds the maximum levels of UFGT activity are achieved by 22 days after pollination (DAP) and then enzyme activity declines. This contrasts with the temporal pattern of gene expression in *Bz* seeds, for which UFGT activity increases, beginning at 30 DAP, and continues to accumulate throughout the remainder of seed development and dehydration [4]. In *Bz* seeds, UFGT activity is proportional to anthocyanin content (Klein, unpublished observations). UFGT is localized in the aleurone in *Bz* seeds; in *bz-m4 Derivative 6856* seeds, enzyme accumulation is shifted from the aleurone to the subaleurone endosperm layers of the seed [5].

Structural genes for the enzymes of anthocyanin synthesis (*A, A2, Bz, Bz2, C2*) are thought to be required for colour formation in all of the tissues of the plant [6, 7]. Different allelic forms of several regulatory genes determine which tissues actually accumulate anthocyanin pigment [6]. For example, lines homozygous for *R-r-std* allele on chromosome 10, develop colour in the aleurone of the seed, the primary root and coleoptile of the seedling, the leaf sheath and anthers. Both Coe (personal communication) and Dooner [5] noted that *bz-m4 Derivative 6856* permits some anthocyanin pigmentation in plant parts (coleoptile, leaf sheath, and anthers). However the actual level of UFGT activity in these plant tissues has not been measured or compared to the expression of the wild-type *Bz* allele.

Both the wild-type *Bz-McC* [8] and *bz-m4 Derivative 6856* (Klein, A. S., *et al.*, in preparation) alleles have been cloned. The mutation in *bz-m4 Derivative 6856* is complex: two *Ds* elements flank a partial duplication of the *bz* locus and this 7 kb transposon-like structure is inserted within the 5'-nontranslated leader of the *Bz* transcriptional unit. The site and size of the *Ds* insertion in *bz-m4 Derivative 6856* suggests it must disrupt expression of the locus.

Gerats and coworkers [9] proposed that a deletion of part of the *Shrunken* (*Sh*, sucrose synthetase 1) locus which occurred during the origin of *bz-m4*, has placed the *bz-m4* under control of *Sh* regulatory sequences. They believe the temporal and tissue-specific pattern of *bz-m4* expression in the endosperm is similar to sucrose synthetase. The expression of *Sh* has been characterized in a number of plant tissues and *Sh* is known to be an anaerobic response gene [10]. One implication of this Gerats' hypothesis is that *bz-m4* expression will be induced by conditions which activate expression of *Sh*.

To more fully understand how the *Ds* insertion in *bz-m4 Derivative 6856* influences gene expression, we characterized UFGT activity in two plant tissues, the coleoptile and primary root. The genetic determinants of anthocyanin pigmentation are well defined for these seedling tissues. Seedlings are relatively easy to grow and to harvest under uniform conditions. Therefore we were able to alter growth conditions to compare the effects of these changes on the expression of the mutant versus the wild-type allele. UFGT activity in *bz-m4* seedlings ranged from 15 to 45% of that of the *Bz-McC* allele. Light activation (phytochrome conversion), is required for the expression of both *bz-m4 Derivative 6856* and *Bz-McC*. Anaerobic treatment does not stimulate the expression of *bz-m4*. The data obtained thus far suggest that while the expression of *bz-m4* is quantitatively reduced in seedling tissues, its developmental expression in these tissues is similar to that of *Bz-McC*.

RESULTS AND DISCUSSION

Levels of expression of different *Bz* alleles were compared for two seedling tissues, the primary root and coleoptile. All lines were homozygous for the *P-ww* allele and for the *R-r-std* allele, which control the pattern of tissue pigmentation. Common allelic forms at *b* locus, which may act as a duplicate factor for the *r* locus, do not appear to influence pigmentation in seedling tissues [10].

Both the *Bz-McC* and the *bz-m4 Derivative 6856* alleles conferred similar levels of red colour to the primary root and coleoptile of the seedlings. By contrast, seedlings homozygous for the *bz-R* reference allele, lacked anthocyanin colour in the coleoptile and exhibited faint bronze colour in the uppermost portion of the primary root.

Styles and Ceska [12] found moderate levels of 3-*O* glycosylated flavonols in extracts of *bz* coleoptiles. They postulated that a second 3-*O*-flavonol glucosyltransferase was active in these seedlings tissues. In our hands, extracts of the primary roots and coleoptiles of seedlings homozygous recessive for the null allele, *bz-R*, had no measurable UFGT activity. If there is a second 3-*O*-flavonol glucosyl transferase in *bz* coleoptiles, it must have substantially different physical properties (stability, pH optima, etc.) than that of the *Bz1* gene product. Therefore, we attributed UFGT activity in extracts of *bz-m4* and *Bz-McC* seedlings to the expression of the *bronze 1* locus.

UFGT activity was at least two-fold higher in primary root of *Bz-McC* seedlings than that of the mutant *bz-m4 Derivative 6856* (Fig. 1). The amount of UFGT in extracts of *bz-m4* coleoptiles was substantially lower than that found in extracts of wild-type material. The variability in extractable UFGT activity between experimental trials (Fig. 1 A, B; Fig. 2) may be due to differences between seed lots and hence germination rates. Consequently the time of optimal sensitivity to light-mediated induction of pigmentation also varied. However the difference in UFGT activity between genotypes was statistically significant ($P < 0.01\%$) in all experiments.

UFGT activity is lower in seedling tissues of both genotypes when compared to the levels of enzyme that are found in extracts of mature seeds: UFGT activity in the *Bz-McC* coleoptile extracts ranged from 0.4 to 0.8 nmol/min/mg protein whereas in extracts from mature seeds (endosperm and aleurone) UFGT activity was on the order of 4.4 nmol/min/mg protein.

Anaerobic stress

Recently *Sh* has been identified as an anaerobic response gene; sucrose synthetase activity is elevated in seedling tissue as a result of anaerobic stress [11]. If the regulatory sequences from *sh* are fused to *bz* coding sequences in *bz-m4* [9] then UFGT activity in seedling roots might be expected to increase as a result of anaerobiosis. To test this hypothesis, *bz-m4* seeds were allowed to germinate normally, were exposed to light for two days to condition anthocyanin gene expression, and were then submerged in 10 mM Tris buffer (pH 7.0) for 48 hr to induce the anaerobic response [13]. The coding sequences for sucrose synthetase are missing in *bz-m4* [14]. Therefore, the effectiveness of the anaerobic treatment was monitored by measuring alcohol dehydrogenase activity (ADH). Elevation of ADH levels is a well-characterised anaerobic response [13]. *Bz-McC* seed were treated in a similar manner to determine whether anaerobic stress influenced wild-type *Bz* gene expression.

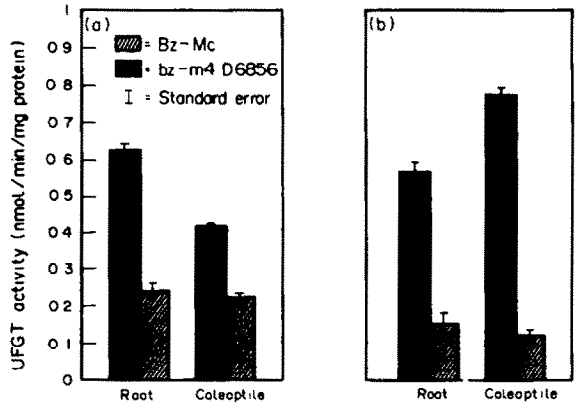

Fig. 1. UFGT levels in seedling tissues. Panel A experimental trial one. Panel B experimental trial two.

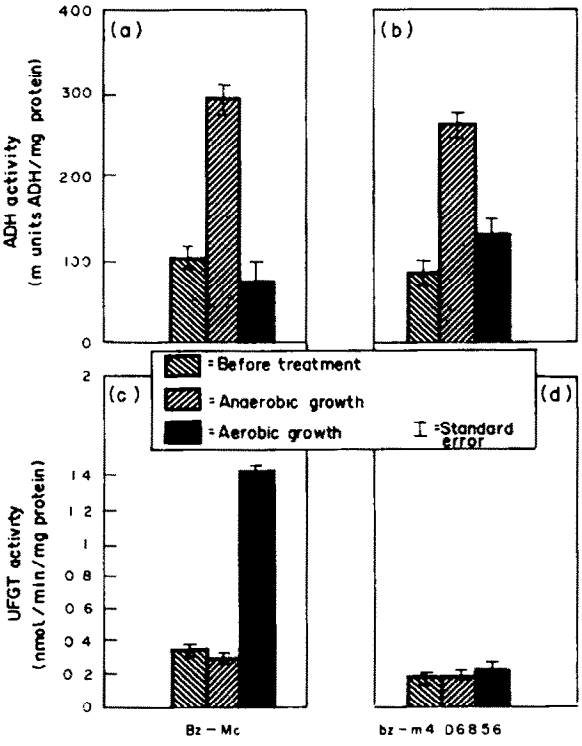

Fig. 2. The effects of anaerobic treatment on seedlings tissues. Panels A, B: accumulation of ADH activity. Panels C, D: accumulation of UFGT activity. Panels A, C: wild-type seedlings. Panels B, D: *bz-m4* seedlings.

ADH activity increased three-fold over pretreatment levels in both *Bz-McC* and *bz-m4* roots (Fig. 2). ADH levels were at least two-fold higher in extracts of anaerobic roots than those from seedlings which were grown for the same length of time under aerobic conditions. Anaerobic treatment did not increase UFGT activity in *bz-m4* seedling roots. These data argue against the involvement of *sh* regulatory sequences in the control of gene expression in the mutant *bz-m4 Derivative 6856*. Anaerobiosis

inhibited UFGT accumulation in wild-type *Bz* roots. Thus it appears that expression of the anthocyanin pathway is not part of the anaerobic response.

Regulatory interactions

Expression of *bz-m4* in the developing seed has an unusual temporal pattern compared by *Bz*. Functional alleles at both the *B* and *C* loci are required for *Bz-McC* expression [15]. In contrast the expression of *bz-m4* is independent of the genic state at *R* and/or *C* [5]. We investigated whether *bz-m4* expression in plant tissues, responds to or requires the function of the same *trans*-acting regulatory genes for required wild-type *Bz* expression.

Plants recessive for the genetic trait *pl* (sunred) develop anthocyanins only where the tissues are exposed to light [6]. *Bz-McC, pl* seedlings were grown in total darkness for four days and harvested under a green safe light. There was no detectible UFGT in extracts from either the root or coleoptile. When *bz-m4* seedlings were germinated under similar conditions, extracts from these tissues also lacked UFGT activity. Since light is required to activate the expression of both alleles in seedling tissues, *bz-m4* may still be responsive to some of the *trans*-acting regulatory factors which are required for *Bz-McC* expression.

In summary, expression of the *Bz* gene product, UFGT, is lower in *bz-m4 Derivative 6856* in both the primary root and coleoptile, than that of the wild-type progenitor *Bz-McC*. Both alleles require light activation for expression. The levels of UFGT in *bz-m4* are still adequate to confer normal anthocyanin accumulation in the seedling. Expression of the *bz-m4* allele in seedling roots is not influenced by anaerobiosis. This result conflicts with the simple model proposed in ref. [9] wherein the regulatory sequences of *sh*, an anaerobic-response gene, controls the expression of UFGT in *bz-m4 Derivative 6856*.

EXPERIMENTAL

Plant materials. Bz-McC is the designation for the wild-type *Bz* allele found in the genetic stocks of Dr Barbara McClintock [5, 16]. The *Ds* transposable element mutation, *bz-m4 Derivative 6856*, was isolated by McClintock [1]. Both *bz* alleles were crossed into a W22 inbred line; near isogenic stocks were produced by repeated backcrossing to the W22 parent. The *Bz-McC* (W22) line was generously provided by Dr Hugo Dooner; the *bz-m4 Derivative 6856* (W22) stock was obtained from Dr Jerry Kermicle. All stocks were homozygous recessive for *sh*. The *Ds*-transposable element mutant was examined in the absence of *Ac*; under these conditions the mutant phenotype is stable both in somatic tissues and in the germ line [4].

Growth conditions. In each experiment, samples consisted of 50 seeds of a given genotype. Seeds were surface sterilized in a 15% chlorox solution for 20 min, rinsed with Milli-Q grade H_2O and spread on a double thick layer of moist teri-towel in glass baking dishes. The seeds were covered with a layer of paper towel and kept moist at all times; the baking dishes were loosely covered with a glass plate to reduce evaporation. The seeds were allowed to germinate in the dark at 28°, for approximately 48 hr, until the primary root was 3–5 cm long and the coleoptile was just breaking through the pericarp. The seedlings were shifted to 21° and maintained under continuous irradience (GE Cool White Fluorescent Bulbs, 0.13 W/m^2) in a growth chamber (Freas model 818, GCA Precision Scientific, Bedford, MA). Substantial anthocyanin coloration developed over the next 72–120 hr. Seedlings were harvested when their coleoptiles were approximately 1.5 cm in length and the primary roots were 7–9 cm in length. Pigmented tissue was collected from the tip of the coleoptile (~0.5 cm) and the ~1 cm of the primary root immediately adjacent to the seed. The samples were frozen in liquid nitrogen and stored at −70°.

Enzyme extractions. Frozen tissue was ground to a fine powder with liquid N_2 in a mortar and pestle. The samples were extracted as described in ref. [17] with minor modifications: Phenylmethylsulphonyl fluoride was omitted from the extraction buffer and the volume of buffer was decreased to 2 ml/g fr. wt.

Enzyme assays. UFGT activity was measured as described by ref. [7] with some modifications: Quercetin was a 3.3 mM soln in MeOH. Each reaction contained: 10–70 μl of enzyme extract, 69.3 nmol of quercetin and 200 nmol UDP glucose in a final volume of 200 μl. The samples were incubated at 37° for 15 min. 100 μl of the upper phase of the Folch partition [18], containing most of the quercetin and isoquecitrin, were separated on a 10 μ Versapack C18 column (Alltech, Applied Science Laboratories, Deerfield IL.) on a Waters Model M6000 HPLC. The eluting solvent was HOAc–MeOH–H_2O (10:20:70) at 3 ml/min. Under these conditions the adjusted retention times were 2.3 min for isoquercitrin and 4.2 quercetin. Product formation was monitored at 254 nm on a Hitachi Model 100-40 spectrophotometer.

Alcohol dehydrogenase activity was measured as described in ref. [19]. Protein was determined by the method of ref. [20] using bovine serum albumin as a standard.

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