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Cloning of a Mutable *bz2* Allele of Maize by Transposon Tagging and Differential Hybridization

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Manuscript received June 15, 1987
Accepted August 14, 1987

ABSTRACT

Sequences of *Bronze2* (*Bz2*), a maize gene which is required for the synthesis of the purple pigment anthocyanin, have been cloned by combining the techniques of transposon tagging and differential hybridization. First, a mutable *bz2* allele (*bz2-mu1*) was recovered from a Mutator line. The mutation was assumed to result from an insertion of the transposable element *Mutator* (*Mu*), which is found in 10–60 copies in Mutator lines. A library was prepared using DNA isolated from *bz2-mu1*, and a small bank of *Mu*-specific candidate clones was selected. Because much is known about the genetic requirements for the synthesis of anthocyanin in different tissues, we were able to identify the *bz2* clone based on its hybridization to RNA isolated from different *bz2* mutants. Only one clone hybridized to RNA isolated from the husks of purple (*Bz2, B, Pl*) plants but not to RNA isolated from the husks of green (*an-bz2-6923, b, pl*) plants. Further confirmation was provided by the hybridization pattern of the clone on blots containing DNA from other *bz2* mutants. We present a restriction map of the clone, indicating the location and orientation of the 1.4-kbp *Mu* insert. We identify the transcribed region, the direction of transcription and the location of the 1.4-kbp *Mu* insert in an independently isolated mutant, *bz2-mu2*.

THE transposable element family *Mutator* (*Mu*) was identified in the Mutator line of maize originally characterized by D. S. ROBERTSON (1978). Unlike other maize transposable element lines, the Mutator line is characterized by non-Mendelian transmission of the Mutator phenotype to nearly all of the progeny, and by a 20–50-fold increase in the spontaneous mutation rate (ROBERTSON and MASCIA 1981). The high rate of recovery of new mutants is a great advantage of the Mutator system. Like insertions of other maize transposable elements, *Mu* insertions are often somatically unstable. Mutable alleles of the genes affecting the synthesis of the purple pigment anthocyanin revert late during kernel development, resulting in spots of anthocyanin-containing cells on a background of unpigmented cells. Because of the ease with which such mutants can be identified, the genes involved in pigmentation have been studied extensively, and the genetic requirements for pigmentation of many tissues is well characterized. The product of one of these genes, *Bronze2* (*Bz2*), is involved in the final steps of anthocyanin synthesis (REDDY and COE 1962).

In an effort to characterize the *Mutator* family of transposable elements, *Mu*-induced mutations have recently been isolated in a number of maize loci. One of these, an unstable allele of *Alcohol dehydrogenase1* (STROMMER *et al.* 1982), contained an insert of 1.4 kbp, called *Mu1* (BENNETZEN *et al.* 1984), which was shown to interrupt transcription. A homologous 1.7-kbp insert (*Mu1.7*) was identified in an unstable *Bronze1* allele (TAYLOR, CHANDLER and WALBOT 1986). Sequences homologous to the internal region of *Mu1* and *Mu1.7* have been shown to exist in 10–60 copies in Mutator lines (ALLEMAN and FREELING 1986; V. WALBOT, unpublished data) and in 1–3 copies in lines of maize that have a normal low mutation rate (CHANDLER, RIVIN and WALBOT 1986). The terminal repeats of *Mu1* and *Mu1.7*, however, show less specificity. They are found in about 60–70 copies in Mutator lines and in about 40 copies in non-Mutator lines (CHANDLER, RIVIN and WALBOT 1986).

Two independent mutants in the *Bz2* gene were recovered from Mutator stocks (WALBOT 1986). The original mutant kernels exhibited the spotted phenotype characteristic of transposable element-induced mutations, but many of their progeny varied in the timing and frequency of somatic reversion. Some lines showed a progressive loss of the spotted phenotype in 1–3 generations. This loss has been shown to correlate with an overall increase in DNA modification of the *Mu* elements present in these lines (CHANDLER and WALBOT 1986). Further characterization of this phenomenon required probes specific to the *Bz2* gene.

Cloning techniques which require knowledge of protein sequence cannot be used to isolate the *Bz2* gene because the product of this gene is unknown. Transposon tagging (BINGHAM, LEVIS and RUBIN 1981) may be used only as a first step because of the many genomic copies of *Mu* present in Mutator lines.

Workers using other maize transposable elements have been able to take advantage of the large differences in DNA modification or in the restriction patterns among the homologous elements present in the genome to identify the active element responsible for the mutant phenotype (FEDOROFF, FURTEK and NELSON 1984; O'REILLY *et al.* 1985; CONE, BURR and BURR 1986; WIENAND *et al.* 1986). Because Mutator lines have so many similar active elements, however, a different method of identifying a *bz2* clone must be used.

Differential hybridization is a useful technique for clone identification when a pattern of hybridization can be predicted. It can be easily adapted to genetically well-characterized alleles, like those involved in anthocyanin biosynthesis, whose expression in various tissues or genetic backgrounds is well understood. In the case of *Bz2,* this technique was facilitated by the existence of a viable, well-characterized *bz2* deletion mutant, *an-bz2-6923* (COE and NEUFFER 1977). This mutant, recovered from material irradiated in atomic bomb tests, has a deletion on the long arm of chromosome *1*, encompassing both the *anther ear* locus (map position 104) and the *bronze2* locus (map position 106). A *Mu*-specific phage clone which contains *bz2* sequences would be expected to hybridize to RNA isolated from plants expressing all the genes required for anthocyanin production, but it would not be expected to hybridize to RNA isolated from the *an-6923* plants. Additional confirmation of *bz2* sequences can be provided by the hybridization pattern of the clone with DNA isolated from other *bz2* mutants.

We report here the identification of a *bz2* clone by a two-step procedure: we used a *Mu*-specific probe to screen a genomic library prepared with DNA isolated from a *Mu*-induced *bz2* mutant, *bz2-mu1*, establishing a small collection of *Mu*-specific clones. The hybridization pattern of these clones to different RNA species was used to identify the presence of *bz2* sequences in one clone. We present a restriction map of the *bz2* region, identify the section of the map that hybridizes to the transcript, and indicate the direction of transcription. We also show the location of the *Mu* insertion in an independently isolated mutant, *bz2-mu2*.

MATERIALS AND METHODS

Stocks: The original Mutator stock used to isolate the *bz2* mutants was a gift from D. S. ROBERTSON; the line contained all of the genes required for anthocyanin pigmentation of the aleurone and is referred to by ROBERTSON as a purple Mutator line (ROBERTSON *et al.* 1985). The *bz2* tester in hybrid W23/K55 background was a gift of E. H. COE. The *bz2-Ds* mutant (*bz2-m*) (NUFFER 1953) and revertant were gifts of DREW SCHWARTZ. The *anther ear bronze2* deletion stock *an-bz2-6923* was obtained from the Maize Genetics Cooperative. The *bz2-mu1* mutant was selected as a spotted kernel (Figure 1) by crossing the purple Mutator stock to the *bz2* tester (WALBOT, BRIGGS and CHANDLER 1985). This mutant was subsequently crossed onto a *bz2* tester stock as a test for allelism. Further crosses shown in Figure 1 were performed in order to propagate the stock. A second mutant, *bz2-mu2*, was not recognized in the original screen because of the heavy spotting pattern on the crown. It was found in 1986 when the ears were shelled. On propagation it showed fine spotting, identical to *bz2-mu1*. Thus the corrected mutation frequency is 3 per 10^5 gametes for *Bz2* in this experiment.

1982	*bz2* x B139-7 (purple Mutator)
W1982	*bz2* x BB2 (*bz2-mu1*)
1983	*bz2* x C230-7
W1983	CF48(x)
1984	D379-14 x *bz2*

FIGURE 1.—Isolation and propagation of the *bz2-mu1* allele. In 1982, the purple Mutator plant B139-7 was crossed onto a *bz2* tester. A spotted kernel resulting from this cross was planted in the winter of 1982 as BB2 and was crossed onto a *bz2* tester. A spotted kernel from this ear was planted in the summer of 1983 as C230-7 and backcrossed to *bz2* tester; progeny were selfed. A spotted kernel from this cross was planted in the summer of 1984 as D379-14 and backcrossed by *bz2* tester. Twenty spotted kernels from this ear were germinated, their DNA was isolated, and it was used to prepare a library.

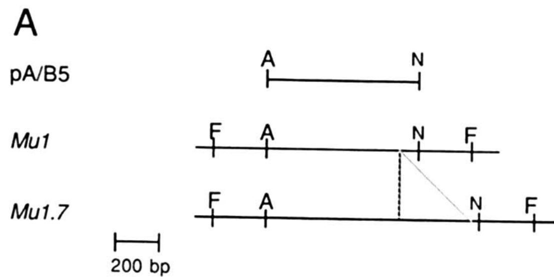
DNA probes and vectors: *Bam*HI-digested λ J1 arms (MULLINS *et al.* 1984) were the gift of VICKI CHANDLER. The pA/B5 probe contains the internal 650-bp *AvaI*/*Bst*NI fragment of *Mu1* (CHANDLER, RIVIN and WALBOT 1986). A *Bz1* coding region probe, pMBZ1P1 (FEDOROFF, FURTEK and NELSON 1984) and a sea urchin actin cDNA, pSA38 (MERLINO *et al.* 1980), were provided by DOUG FURTEK and KENT PETERS, respectively. M13 subclones of pP420 were prepared by KEN LUEHRSEN.

Reagents: Restriction enzymes, T4 DNA Ligase, DNA Polymerase I and DNA Polymerase Large Fragment were obtained from either BRL or New England Biolabs and used according to the manufacturers' recommendations.

Isolation of plasmid, phage and plant DNA: Plasmids and phage DNAs were isolated by standard procedures (ISH-HOROWICZ and BURKE 1981; MANIATIS, FRITSCH and SAMBROOK 1982; HELMS *et al.* 1985). Maize genomic DNA was prepared from individual 5-day etiolated seedlings (DELLAPORTA, WOOD and HICKS 1983) or from immature cobs by centrifugation in CsCl/ethidium bromide (RIVIN, ZIMMER and WALBOT 1982).

Southern blot analysis: Southern blots (MEINKOTH and WAHL 1984) were done using either nitrocellulose (Schleicher & Schuell), Genetran (Plasco) or Hybond-N (Amersham). In the case of nylon membranes, the blots were washed (60°, 30 min) in 0.1% SDS, 0.1 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) after baking and before prehybridization. Nick-translated probes (RIGBY *et al.* 1977) and random primer labeled probes (FEINBERG and VOGELSTEIN 1983) were separated from unincorporated nucleotides by spin dialysis (NEAL and FLORINI 1973).

Cloning of maize DNA and isolation of *Mu*-specific sequences: Genomic DNA was isolated from pooled seedlings because reasonable amounts of material from single plants were not available. A partial *Sau*3A digest of genomic DNA was size-fractionated on a 1.5–5 M NaCl gradient (180,000 × *g*, 3 hr, 18°). Fragments of 10–20 kbp were ligated to purified, *Bam*HI-digested, λ J1 arms, packaged (using extracts prepared from BHB2688 and BHB2690)



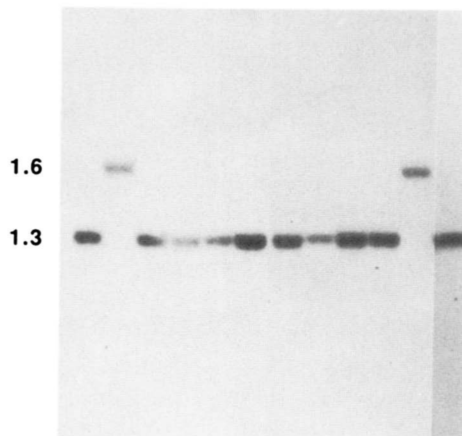


FIGURE 2.—Hybridization of 12 *Mu*-specific phage clones with the pA/B5 probe. (A) simplified map of the endogenous *Mu* elements *Mu1* and *Mu1.7* and the clone pA/B5. The restriction enzymes used in this study are: A, *Ava*I; N, *Bst*NI; H, *Hin*fI. (B) phage DNA digested with *Hin*fI, electrophoresed through 1% agarose, transferred to nitrocellulose and hybridized to pA/B5 as described in MATERIALS AND METHODS. Each lane contains DNA from a unique clone; the fifth lane contains DNA isolated from clone #15. The variation in hybridization intensity reflects the variation in the amounts of DNA loaded on the gel.

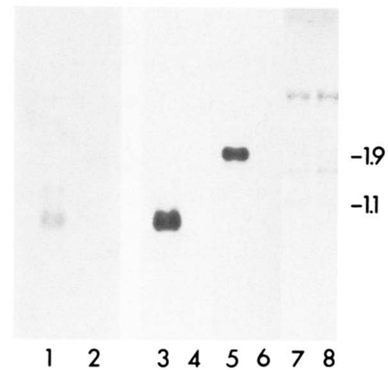


FIGURE 3.—Specific hybridization of clone #15 to husk RNA. Two micrograms of poly(A) RNA were electrophoresed through 1.5% agarose formaldehyde gels and blotted and hybridized as described. Lanes 1, 3, 5 and 7: RNA isolated from purple husks (*Bz2, B, Pl*); lanes 2, 4, 6 and 8: RNA isolated from green husks (*an-bz2-6923, b, pl*). The probes were: lanes 1 and 2: clone #15; lanes 3 and 4: pP300, a subclone of #15; lanes 5 and 6: pBZ1P1, a *Bz1* coding region probe; lanes 7 and 8: pSA38, a sea urchin actin cDNA. Size standards are marked in kilobase pairs.

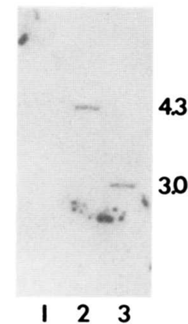


FIGURE 4.—Hybridization of subclone pP300 to DNA from *bz2* mutants. Five micrograms per lane of genomic DNA prepared from immature cobs was digested to completion with *Bgl*II, electrophoresed through 1% agarose, blotted to Genetran and hybridized to probe pP300. Lane 1: *an-6923* DNA; lane 2: *bz2-m* DNA and lane 3: *bz2-m* revertant DNA. Sizes are marked in kilobase pairs.

(MANIATIS, FRITSCH and SAMBROOK 1982) and used to infect *Escherichia coli* A575. The resulting 1.5 million plaques were screened by filter hybridization (BENTON and DAVIS 1977) using the pA/B5 probe.

RNA isolation and Northern blot analysis: RNA was isolated from freshly harvested husks (CHIRGWIN *et al.* 1979) and enriched for poly(A) RNA (AVIV and LEDER 1972) using oligo(dT)-cellulose chromatography (Collaborative Research). Two micrograms were electrophoresed through formaldehyde agarose gels (POTTER *et al.* 1981). The gels were washed in 40 mM NaOH, 30 min, followed by 2 M ammonium acetate (pH 7), 30 min. After blotting onto Genetran (Plasco) using 20 × SSC, the Northern blots were probed (MEINKOTH and WAHL 1984).

RESULTS

The *bz2* mutants we had isolated were likely to contain *Mu* insertions because they showed the characteristic unstable phenotype and were derived from a known Mutator stock. In order to simplify the later identification of the clone, the *Mu* copy number in many progeny of the original *bz2-mu1* kernel was estimated by Southern blot analysis, using a *Mu*-specific sequence, pA/B5 (Figure 2), as a probe. DNA isolated from those with the fewest copies of *Mu* was pooled and used to prepare a library (20 etiolated seedlings grown from sibling spotted kernels, ear D379-14 × 386, Figure 1). The *Mu* copy number in these genomic DNAs was approximately 12–15.

Twenty-five clones which strongly hybridized to the pA/B5 probe were selected from a library of 1.5 million plaques. Of these, the 20 clones which grew well were chosen for further analysis. DNA was extracted from the purified phages, digested with *Hin*fI, and subjected to Southern blot analysis with the pA/B5 probe. Each of the phage clones had a hybridizing band at 1.3 or 1.6 kbp characteristic of *Mu* elements (Figure 2).

Fourteen of the *Mu*-specific phage clones were further distinguished by their hybridization patterns to two RNA species by Northern analysis. The first RNA

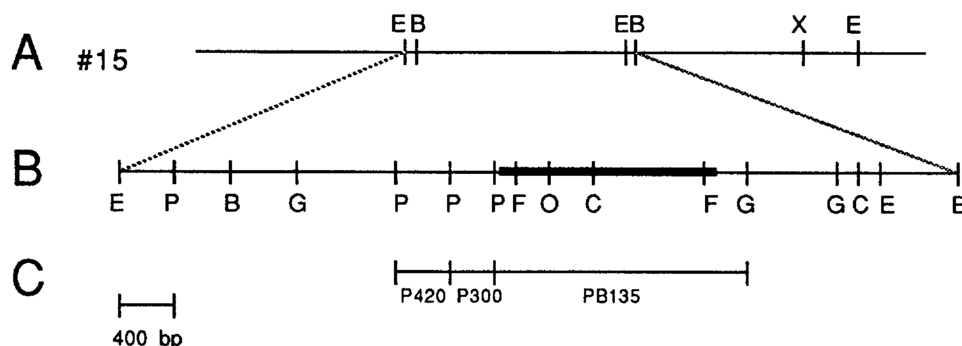


FIGURE 5.—Restriction map of clone #15 and subclones. (A) A simplified map of the 13-kbp insert in clone #15 (not drawn to scale). (B) An expansion of the region of #15 which includes the *Mu* insertion (represented by the heavy line) and the region which hybridizes to purple husk RNA. The orientation of the *Mu* insert is marked by the asymmetric sites *Not*I and *Nco*I. (C) The subclones which hybridize to the 850 base RNA in purple husk. The restriction enzymes used in the study are: B, *Bam*HI; C, *Nco*I; E, *Eco*RI; F, *Hin*fI; G, *Bgl*II; O, *Not*I; and P, *Pst*I.

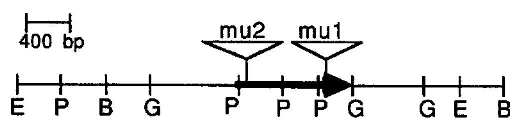


FIGURE 6.—A map of the *Bz2* region, showing locations of the 1.4-kbp *Mu* insertions and direction of transcription. The insertion in *bz2-mu2* was determined by genomic Southern blots using the subclones shown in Figure 5 as probes. The direction of transcription was determined by parallel Northern blots using single-stranded complementary probes from the coding region (pP420). (Insertions not drawn to scale.)

preparation (Figure 3, lanes 1, 3, 5 and 7) was isolated from the purple husks of plants which contained all the genes required for anthocyanin production in plant tissues (COE and NEUFFER 1977). The second RNA preparation (Figure 3, lanes 2, 4, 6 and 8) was isolated from husks of plants carrying alleles which do not permit the synthesis of anthocyanin in husks (*b*, *pl*) in addition to carrying a deletion in the *Bz2* locus (*an-bz2-6923*). This RNA preparation, therefore, was not expected to contain *Bz2* sequences. Six of the nick-translated phage DNAs hybridized to neither RNA species; seven hybridized to both (data not shown). DNA from only one of the tested phage clones, #15, hybridized to the RNA from purple husks but not to the RNA from husks of the deletion mutant (Figure 3, lanes 1 and 2). When a restriction fragment (pP300, Figure 5) adjacent to the *Mu* insertion in #15 was used as a probe, the same differential pattern of hybridization was obtained (Figure 3, lanes 3 and 4). A control probe containing *Bz1* coding sequences, pMBZ1P1, also hybridized differentially to the RNA preparations, confirming that the regulatory alleles *B* and *Pl* are required for transcription of *Bz1* (lanes 5 and 6). A second control probe, a sea urchin actin cDNA probe, pSA38, hybridized equally to both husk RNA preparations (lanes 7 and 8). On the basis of the pattern of differential hybridization, we identified clone #15 as a *bz2* clone.

Further evidence that clone #15 contains *bz2* sequences is provided by its hybridization pattern to DNA isolated from a *bz2-Ds* mutant (*bz2-m*) and its revertant. The reversion presumably results from the excision of the *Ds* transposable element present in the *bz2-m* allele. A probe containing *bz2* sequences should detect this size difference on a Southern blot. Indeed the pP300 probe hybridizes to a 4.3-kbp *Bgl*II fragment in the DNA isolated from *bz2-m* and to a 3.0-kbp *Bgl*II fragment in the DNA isolated from the revertant (Figure 4, lanes 2 and 3). In addition the pP300 probe does not hybridize to DNA isolated from the *an-bz2-6923* deletion mutant (Figure 4, lane 1).

The only RNA species detected by this probe is approximately 850 bases long. Several subclones (Figure 5) adjacent to the *Mu* insert in clone #15 hybridize to this RNA (data not shown). Flanking subclones do not hybridize. The direction of transcription was inferred by the hybridization of single-stranded coding region probes (pP420, subcloned in both directions in M13mp19).

Another *Mu* insertion into the *Bz2* locus, *bz2-mu2*, has been mapped using the subclones shown in Figure 5 as probes on genomic Southern blots. This mutant also has a 1.4-kbp insertion within the transcribed region, about 700 bp upstream from the insertion in the *bz2-mu1* isolate (Figure 6). Both insertions are in the same orientation.

DISCUSSION

We have cloned the *bz2-mu1* locus by a two-step procedure using transposon tagging and differential hybridization. *Mu*-specific clones were selected from a library prepared using DNA isolated from the mutant *bz2-mu1*. One of the *Mu*-specific clones was identified as a *bz2* clone because it hybridized to RNA isolated from purple husks (*Bz2, B, Pl*) but not to husk RNA from plants carrying both a deletion encompassing the *Bz2* region (*an-bz2-6923*) and alleles which do not permit the synthesis of anthocyanin in husks (*b, pl*). Further confirmation was provided by the hybridization pattern of a subclone with different *bz2*

mutants. One of these mutant alleles, the *bz2-Ds* allele, has also been recently cloned (THERES, SCHEELE and STARLINGER 1987).

Clone #15 contains at least part, and perhaps all, of the *bz2* transcribed region. Three adjacent subclones hybridize to the 850 base RNA, while flanking subclones do not. The 1.4-kbp *Mu1* elements inserted in *bz2-mu1* and *bz2-mu2* are 700 bp apart and both are found within the transcribed region. Although the insertions are in different locations, they are in the same orientation and upon propagation result in an identical phenotype of fine dark spots. Interestingly, the *Mu* element orientation is the same relative to transcription initiation in these *bz2* mutable alleles as in the four other *Mu*-induced mutables examined thus far (BENNETZEN *et al.* 1984; O'REILLY *et al.* 1985; TAYLOR, CHANDLER and WALBOT 1986).

Differential hybridization has been used to clone many genes whose expression is well characterized. Usually, a library is screened in duplicate and probed with two cDNA preparations, only one of which contains copies of the transcript of interest. The method has been extended to low abundance messages through the use of subtractive probes (DAVIS 1986). These techniques could be easily adapted to the cloning of genes for which developmentally regulated or null mutants exist. But if another technique can be used as an initial screen, *e.g.*, transposon tagging, the resulting collection of clones is small enough that their hybridization properties may be examined by Northern analysis. This allows the technique to be extended to genes for which the available mutants are not so well characterized. Any change in the level of transcription or the pattern of transcripts will be detected.

All 20 of the phage clones which hybridized to the *Mu*-specific probe contained either a 1.3- or 1.6-kbp homologous *Hin*fI fragment characteristic of *Mu1* and *Mu1.7* elements. Of the 14 unique clones used as probes on Northern blots, eight (including #15, the *bz2* clone) hybridized to RNA. This is a surprisingly high proportion. It may be that Mutator elements (at least those elements homologous to the 650-bp internal region of *Mu*) preferentially transpose to actively transcribed regions. Such a mechanism has already been proposed (BENNETZEN 1984) to account for the high mutation rate in Mutator lines of maize.

We would like to thank V. CHANDLER, L. TAYLOR and J. EBERWINE for helpful discussions, C. WARREN for technical assistance and A. BLOOM for manuscript preparation. This research was supported by a grant from the National Institutes of Health (GM 32422). M.M. was supported by a National Science Foundation Postdoctoral Fellowship in Plant Biology.

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Communicating editor: B. BURR