Theor Appl Genet (1987) 74:471–475

Stability of deletion, insertion and point mutations at the *bronze* locus in maize

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Received November 17, 1986; Accepted March 27, 1987
Communicated by P. Maliga

Summary. Phenotypic revertants from several kinds of mutations, including deletions, have been detected by pollen analysis at the *wx* and *Adh* loci in maize. Mutations in these genes give phenotypic revertants with median frequencies of 0.7 and 0.5×10^{-5}, respectively. However, the nature of such revertants can only be analyzed following their recovery from conventional matings. In the current study large seed populations derived from crosses involving several *bz* (*bronze*) locus mutations in maize were examined for reversion to a *Bz* (purple) expression. Deletion, insertion and point mutations were included in the study. Principally, over 2 million gametes of the *bz-R* mutation, which is shown here to be associated with a 340 base pair deletion within the transcribed region of the gene, have been screened for reversion. No revertants from it or any of the other *bz* mutations have been recovered, even though a total of almost 5 million gametes from homoallelic crosses have been examined to date. Results from seed analysis are discussed in reference to those from pollen analysis in maize.

Key words: *Zea mays* – *bronze* locus – Reversion – Stable mutations – Molecular cloning

Introduction

The study of spontaneous mutation in plants was pioneered by L. J. Stadler, who described such endeavor as being "laborious, at best". To make this study easier, he developed an isolation plot technique for determining the spontaneous mutation frequency of specific genes in maize that is practicable for mutation frequencies ranging as low as about 1×10^{-6} (Stadler 1942). Geneticists have used this technique throughout the years to determine the forward mutation frequencies of several genes. Rarely, however, have large populations of gametes been examined for reversion by this procedure.

Nelson (1968) made ingenious use of pollen analysis to study recombination and reversion at the *wx* locus in maize. He found that many homoallelic *wx* stocks gave rise to Wx-staining pollen grains at frequencies in the order of 10^{-5}. Recently, Wessler and Varagona (1985) reported that two such mutants (B and C4) have deletions within or including the *Wx* transcription unit and, therefore, would not be expected to back-mutate or revert as a consequence of suppressor mutations. Freeling (1976) adopted Nelson's prototype pollen analysis in his investigations of intragenic recombination in the *Adh1* locus of maize. He reported a median Adh^+ frequency in homoallelic Adh^- combinations of 0.57×10^{-5}, noting that the revertant frequencies measured in the pollen appeared surprisingly high. In fact, the $Adh^- \rightarrow Adh^+$ reversion frequency exceeded the $Adh^+ \rightarrow Adh^-$ forward mutation frequency as scored in pollen grains. As is true of *wx*, deletion mutations of *Adh* also give phenotypically revertant pollen (Strommer et al. 1982; Taylor and Walbot 1985).

The *wx* and Adh^- mutations discussed above are stable, i.e., they do not revert at the high frequencies (10^{-2}–10^{-3}) usually associated with excision of a transposable element. The apparenty occurrence of reversions from stable mutations, particularly deletions, is intriguing. It is conceivable that the revertant *Wx* and Adh^+ pollen grains arise from activation of or gene conversion with a second locus, but since pollen analysis is destructive such hypotheses cannot be tested. In order to analyze the nature of reversion events, it is necessary to be able to recover individual revertants

from among a large number of mutant sibs. The *bronze* locus in maize provides a particularly suitable system for such analysis. It confers an easily scorable endosperm color phenotype, so large seed populations can be readily screened. In addition, since the locus has been cloned molecularly, any mutation being studied can be characterized precisely. In this paper we report molecular data showing that the spontaneous mutation *bz-R*, that served originally to define the *bronze* locus genetically (Rhoades 1952), is a small deletion within the *bronze* transcribed region. We present reversion data for this deletion mutation and other molecularly defined mutations from studies involving a population of almost 2,5 million seed generated by Stadler's isolation plot technique and discuss this result in light of previous findings in maize.

Materials and methods

Stocks

The *bronze* locus of maize specifies a flavonoid glucosyltransferase (UFGT) which catalyzes one of the last steps in anthocyanin biosynthesis (Larson and Coe 1977; Dooner and Nelson 1977a). In the presence of a functional enzyme (*Bz* genotypes), a purple pigment accumulates in plant parts competent to form anthocyanins. In the absence of a functional enzyme (*bz* genotypes), the pigment is bronze; hence, the name of the gene. The alleles used in this study and the phenotypes they condition are described below.

bz-R (bronze). The *bz-R* mutation makes no detectable UFGT ($UFGT^-$) or cross-reacting material (CRM^-) throughout endosperm development (Dooner and Nelson 1977b). Our *bz-R* allele was obtained originally from J. L. Kermicle in a W22 stock derived by R. A. Brink. It is the stable reference allele at the locus and, from its pedigree, very likely the same as the spontaneous mutation described by Rhoades (1952). Our stock traces back to *bz* stocks sent by M. M. Rhoades to B. McClintock and J. Laughnan in 1946 and 1949, respectively (J. L. Kermicle, B. McClintock and S. Gabay-Laughnan, pers. commun.). Therefore, we are reasonably sure that our *bz-R* allele is the one originally described by Rhoades, though we could not confirm this directly since Dr. Rhoades (pers. commun.) was not able to find a stock that definitely traced back of the original *bz* mutation.

bz-m1 (bronze in the absence of the autonomous transposable element Ac; bronze-purple variegation in its presence). An allele arising from insertion of a 1.1-kb *Ds* element close to the 5′ end of the *Bz-McC* isoallele (McClintock 1951; Dooner et al. 1985).

bz-m2 (bronze-purple variegation). An allele arising from insertion of a 4.5-kb *Ac* element in the middle of the transcribed region of *Bz-McC* (McClintock 1955; Dooner et al. 1985).

bz-m2 (DI) and bz-m2 (DII) (bronze in the absence of Ac; variegated in its presence). The first and second derivatives from *bz-m2* harboring, respectively, 3.3-kb and 3.7-kb *Ds* elements at the locus as a consequence of internal deletion mutations from *Ac* (McClintock 1962; Dooner et al. 1985; Schiefelbein et al. 1985; Dooner et al. 1986).

bz-m4 (D6856) (bronze in the absence of Ac; variegated in its presence). An allele arising from insertion of an approximately 6.8-kb *Ds* element close to the 5′ end of the *Bz-McC* allele (McClintock 1956; Dooner, unpubl. obs.).

bz-E2 and bz-E5 (bronze). Ethylmethane sulfonate (EMS)-induced mutants from the isoallele *Bz-W22*. These mutations are $UFGT^-$, CRM^+ and map in the middle of the transcribed region (Dooner 1986).

Genetic analysis

The advantages of using *r bz* double mutants in recombination and reversion studies of the *bronze* locus and the selection and analysis of purple kernel derivatives from *bz* homoallelic and heteroallelic combinations have been presented (Dooner and Kermicle 1986).

Molecular analysis

Restriction enzyme usage, DNA isolation and phage library construction were carried out as described previously (Dooner et al. 1985). The phage library was constructed in EMBL4 using partially *Sau*3AI-digested DNA from a maize stock carrying the *bz-R* allele. Phage were screened with pAGS 528, the *Kpn*I-*Pst*I fragment internal to the transcribed region of *Bz-McC*, A 6 kb *Eco*RI-*Hind*III fragment from one of the hybridizing phages was subcloned into pUC18 and analysed by restriction mapping and Southern blots. Overlapping fragments of the *bz-R* allele obtained by *Bal*31 deletions (Dean et al. 1985) were subcloned into M13 phage (Messing et al. 1981) and sequenced using the dideoxy method (Sanger et al. 1977).

Results

Reversion of bz mutations

Throughout the years we have accumulated substantial data from experiments designed to test reversion of different *bz* alleles from a bronze to a wild-type purple expression (Dooner 1986). The largest body of data has been generated for the reference *bz-R* allele carried by a *sh bz-R wx* stock which has been used extensively as tester in crosses with homoallelic chemically-induced and transposable element *bz* mutations. The stability of the *bz-R* allele in both male and female gametes is presented in Table 1. In over 2 million male gametes tested we have never seen an instance of reversion of *bz-R* to *Bz*.

Neither did we see reversion of any of the other *bz* alleles in the corresponding population of female gametes. Table 1 summarizes data for those insertions and chemically-induced mutants where populations larger than 200,000 gametes have been screened for reversion. Four *Ds* insertion mutations, *bz-m1, bz-m2 (DI), bz-m2 (DII)* and *bz-m4 (D6856),* and two chemically induced mutations, *bz-E2* and *bz-E5,* are included in the table. The *Ds* insertion mutations are known to revert in the presence of the autonomous element *Ac*. Homozygous stocks not carrying *Ac* were used to generate the data presented in Table 1. The

EMS-induced mutations lack UFGT activity but make normal amounts of an inactive protein. They map in the middle of the transcribed region (Dooner 1986) and most likely represent point mutations in the coding region. We did not obtain revertants from any mutation examined. If any revertant had been recovered, we would have been able to identify the reverting parental allele on the basis of flanking marker composition and restriction site polymorphisms since the progenitor alleles of the insertion mutations (*Bz-McC*) and of the point mutations (*Bz-W22*) have been cloned and characterized (Ralston et al. unpublished).

In a smaller experiment, reversion of *bz-R* through the female germ line was tested by crossing *Sh bz-R Wx; r-r* homozygotes by a *sh bz-R wx; R-r* tester in an isolated detasselling plot. Putative *Bz* reversions would have appeared as mottled purple kernels in otherwise *bronze* ears. None were found in a population of 152,200 gametes tested. Thus, *bz-R* behaves as a stable mutation in both the male and female germ lines. In all, a total of almost 5 million gametes from *bz* homoallelic combinations have been examined to date, but no confirmed cases of reversion have been recovered.

Table 1. Reversion to *Bz* of various *bz* alleles

Allele	Type of gamete	Gamete population	No. of Bz	Reversion frequencies ($\times 10^{-5}$) 95% confidence limits	
				Lower	Upper
bz-R	Male	2,495,900	0	0	0.1
	Female	152,200	0	0	2.4
bz-m1	Female	339,900	0	0	1.1
bz-m2 (DI)	Female	509,500	0	0	0.7
bz-m2 (DII)	Female	404,600	0	0	0.9
bz-m4 (6856)	Female	228,100	0	0	1.6
bz-E2	Female	256,600	0	0	1.4
bz-E5	Female	313,800	0	0	1.2
Other *bz* mutants	Female	443,400	0	0	0.8
Total		4,991,800	0	0	<0.1

Isolation and molecular characterization of a bz-R clone

In order to examine the molecular basis of the *bz-R* mutation, this allele was isolated from a maize EMBL 4 lambda library screened with a *bronze*-specific probe, pAGS528 (Dooner et al. 1985). Two phages isolated from a library of approximately 1×10^6 phages were shown by restriction mapping to contain overlapping fragments of maize DNA. These clones yielded *bronze* fragments of the same size as expected from genomic Southern analysis of *bz-R* maize lines (data not shown). A 6 kb *Eco*RI-*Hind*III fragment, containing the entire *bronze* gene, was subcloned from one of these phages into pUC18 and analyzed further.

The restriction map of the *bz-R* allele was compared to that of the previously cloned *bronze* allele *Bz-McC* (Dooner et al. 1985). Within the 3′ side of the transcribed region, there was a good correlation between the restriction sites in both alleles, except that *bz-R* was found to have an approximately 350 bp smaller *Sac*I-*Pvu*II fragment, suggesting a deletion (Fig. 1). On the 5′ side of the transcribed region and extending 1.5 kb outside of the transcribed region, there was poor correspondence between restriction sites in the two alleles.

To verify the internal deletion and to determine the source of the apparent scrambling of the 5′ end of *bz-R*, this region was sequenced and compared with our unpublished sequence of the *Bz-McC* allele. The deletion within the transcribed region suggested by restriction analysis was verified as a 340 base pair gap. Figure 2 shows the sequence of *Bz-McC* and *bz-R* in the region spanning the deletion. There is a sharp break at the 5′ end of the deletion, while at the 3′ end of the

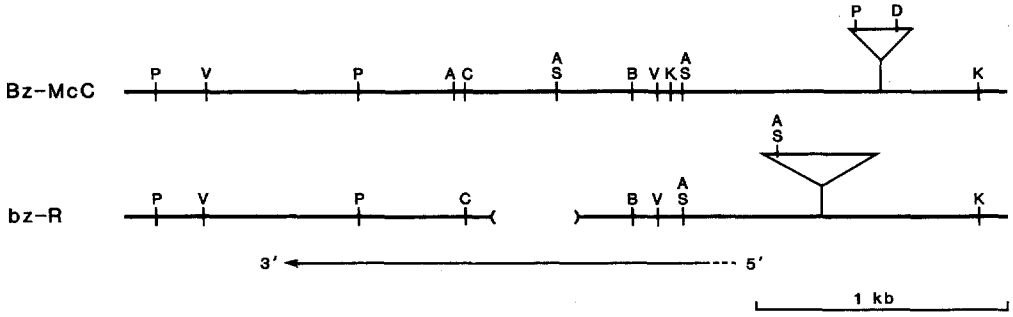

Fig. 1. Restriction map of the *Bz-McC* and *bz-R* alleles oriented in the same direction as the genetic map of *9S*, i.e., with the centromere to the right. (A = *Ava*I, B = *Bst*EII, C = *Sac*I, D = *Dra*I, K = *Kpn*I, P = *Pst*I, V = *Pvu*II, S = *Sma*I). The arrow subtends the transcribed region and indicates the direction of transcription (Dooner et al. 1985). The presence of the insertions shown upstream of the *bronze* transcribed region in the two alleles was determined by DNA sequence analysis

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Bz-McC      CAGCCGTGCG GGTCGGCTGG <TGCGGCGGT GTCGGCGTCG TCCTCGGCGA GGAGGAGGTG GTAGGGGCCG AACGGGACGC AGTTGGGCAG
bz-R        CAGCCGTGCG GGTCGGCTGG <ACGCC

GATCTCCGCG AGGCGCCGCG GTGACGTCGG GCGGGTCCAG GCCTGGGAAC GTGTTGAGTG CCACGGCGGC GGCAGAGCGC GGGAGGCACT GCCCCATGCG

GTGGACGAGG AGGTTGATGA CGTAGTTGAA GTCGCCGGAG ACGACGCCGT CTGGGAGGTC ACGGACGCGG TAGCTGGCGA GGCCCGGGTG GGAGATCAGT

AGCCCGTCCA CCCTGTTTGC TGCTGCGAAC GATGGAAATG CAACAGCCAT TCGATCATCA AACCCGCGCG CAATGA>AGG GACAAGGAAG GGAGAGAAGT
                                                                                        >AGG GACAAGGAAG GGAGAGAAGT
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Fig. 2. Comparison between the nucleotide sequence of *Bz-McC* and *bz-R* in the region spanning the *bz-R* deletion endpoints. The deleted *SmaI* site is underlined. The sequence is presented in the same orientation as the restriction map of Fig. 1, i.e., with the 5′ or centromere end of *bz* to the right

deletion five nucleotides have been oddly replaced by their complements.

Restriction site differences within the transcribed region were found to be due to single base pair changes or to deletion loss. These observations differ from those reported for the *sh* (Burr et al. 1983) and *Adh* (Johns et al. 1983) loci in maize, where no restriction site changes were observed inside the transcribed region of the gene. Outside the *bronze* transcribed region, the differences were due to both single base pair changes and to regions that showed no homology between the two alleles. A map based on restriction analysis and sequencing is presented in Fig. 1. The apparent scrambling in the 5′ region can be resolved into insertions of 265 and 438 base pairs in *Bz-McC* and *bz-R*, respectively. These insertions, which have structures suggestive of transposable elements, are upstream of the *bronze* transcribed region defined by a full-length cDNA clone (unpublished).

Discussion

In this study we have addressed the issue of reversion of non-variegating mutations in plants. Reversion frequencies in the order of 10^{-5}, even for deletion mutants, have been measured by pollen analysis of the *wx* and *Adh* loci in maize. Since pollen analysis is destructive, one cannot recover these reported reversion events to elucidate their nature. One can attempt to overcome this limitation by studying reversion of a gene conditioning an easily scorable seed phenotype. We have examined reversion at the *bronze* locus in maize, a gene that affects anthocyanin pigmentation in many parts of the plant, including the seed. Rare intragenic recombination events occurring with a frequency of 10^{-5} can be detected using this system (Dooner 1986).

The *bronze* mutation for which we have the most extensive data is the reference allele *bz-R*, a mutation not previously characterized molecularly. We have shown here that the *bz-R* spontaneous mutation is associated with a deletion of 340 base pairs within the *bronze* transcribed region, of which almost 300 bp are from the second exon of the gene. Therefore, the deletion appears to be a sufficient cause of the mutation. It is not possible to elucidate the mechanism of origin of the deletion in *bz-R* because its progenitor allele is unknown. However, an odd rearrangement stands out at at the 3′ end of the deletion: 5 bases of the second exon have been replaced by their complements. An inversion of the pentanucleotide sequence relative to the wild-type allele, would have resulted in the reverse complementary sequence. We know of no mechanism by which the observed rearrangement – which represents a change in strand polarity – could have taken place.

We have accumulated substantial data regarding the genetic stability of *bz-R* and various other *bronze* locus mutations. The *bz-R* allele has never been found to revert even though a population in excess of 2 million male gametes, one of the largest derived by conventional crosses in maize, has been screened for reversion. Since *bz-R* is a deficiency of 340 bp, it would not have been expected to back-mutate, but reversion due to activation or conversion of a second locus could have occurred. Had the *bz-R* mutation reverted to a Bz expression in pollen with a frequency similar to the median frequencies reported in pollen for *wx* (Nelson 1968) or *Adh*$^-$ (Freeling 1976), we would have expected from 13 to 18 purple kernel derivatives in the population screened. Yet, none were found. No reversions have been found either among female gametes from several other *bz* mutations examined, including both insertions and point mutations (Table 1; Dooner 1986). When the data for all the *bz* alleles are considered, almost 5 million gametes from homoallelic combinations have been screened to date, but no purple revertants have been obtained. In another study involving large seed populations, no reversions to *R* were recovered among 1,5 million female gametes derived from four spontaneous *r* mutations that retained both closely linked flanking *I-sr* markers present in the progenitor *R* allele (Dooner 1971).

A mutation due to a deletion or an immobile insertion could revert genetically by activation of a second locus that specifies the same or a similar func-

tion or by non-allelic gene conversion with such a locus if the two share homology. Though the former type of event is expected to be very infrequent, the latter, i.e., meiotic gene conversion between duplicate genes on non-homologous chromosomes, has been reported to occur in yeast at a surprisingly high frequency (Jinks-Robertson and Petes 1985). Loci that are functionally related to the four genes being considered, *wx, Adh1, bz,* and *R,* are known to exist in maize: *wx* or starch-bound starch synthase (Nelson et al. 1978), *Adh* (Freeling and Schwartz 1973), *bz* or UFGT (Dooner 1979), *R* (Styles et al. 1973). It is indeed possible that the *Wx* and Adh^+ revertants found by pollen analysis are due to non-allelic gene conversion with the second genes for starch synthase and ADH, particularly since, in the case of ADH, *Adh1* and *Adh2* have been shown to share structural homology (Dennis et al. 1985). If so, non-allelic conversion events are considerably less frequent for *bz* and *R.* Alternatively, the low level reversion of stable (nonmutable) mutations measured in pollen has a nongenetic basis and true genotypic reversions of such mutations in plants are extremely rare events. Discrimination between these alternatives awaits the recovery and analysis of reversions in a system that, like *wx* or *Adh,* is well characterized genetically and molecularly.

Acknowledgements. We would like to extend our sincere appreciation to Jerry Kermicle, Barbara McClintock, Marcus Rhoades, Susan Gabay-Laughnan and John Laughnan for taking the time to trace back stocks grown over a combined period of almost 40 years. That they succeeded in helping us trace the origin of our *bz-R* stock is a tribute to the care with which maize geneticists have kept pedigree records throughout the years. This is paper number 5-4 from Advanced Genetic Sciences.

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