Plant Physiol. (1997) 113: 93–102

Expression and RNA Splicing of the Maize Glutathione S-Transferase *Bronze2* Gene Is Regulated by Cadmium and Other Stresses[1]

Kathleen A. Marrs[2] and Virginia Walbot*

Department of Biological Sciences, Stanford University, Stanford, California 94305–5020

The *Bronze2* (*Bz2*) gene in maize (*Zea mays*) encodes a glutathione S-transferase that performs the last genetically defined step in anthocyanin biosynthesis—tagging anthocyanin precursors with glutathione, allowing for recognition and entry of anthocyanins into the vacuole. Here we show that *Bz2* gene expression is highly induced by heavy metals such as cadmium. Treatment of maize seedlings with cadmium results in a 20-fold increase in *Bz2* message accumulation and a 50-fold increase in the presence of the unspliced, intron-containing transcript. The increase in message levels during cadmium stress appears to result, at least in part, from activation of an alternative mRNA start site approximately 200 nucleotides upstream of the normal start site; this site is not used in unstressed or heat-stressed tissues. The effect of cadmium on the RNA splicing of *Bz2* seems to be specific: splicing of other intron-containing maize genes, including a maize actin gene under the control of the cadmium-inducible *Bz2* promoter, is unaffected by cadmium stress. Conversely, *Bz2* intron splicing is not affected by other stress conditions that induce *Bz2* gene expression, such as abscisic acid, auxin, or cold stress. Surprisingly, the increase in *Bz2* mRNA during cadmium stress does not result in an increase in *Bz2* glutathione S-transferase activity. We propose that an alternative protein may be encoded by *Bz2* that has a role during responses to heavy metals.

Specific enzymatic and regulatory roles have been assigned for most genes of the anthocyanin pathway in maize (*Zea mays*) as a result of genetic and biochemical analysis. *Bz2* is one of the last structural genes required for the production of anthocyanin pigmentation in maize (Coe et al., 1988; Holton and Cornish, 1995). In *bz2* mutants cytoplasmic synthesized anthocyanin precursors are unable to reach their final destination in the vacuole. This inappropriate cytoplasmic accumulation of the precursor pigment cyanidin-3-glucoside causes tissues to develop a bronze color as a result of oxidation and condensation reactions that are poorly understood (Stafford, 1990). These oxidized secondary metabolites in the cytoplasm of *bz2* mutants are somewhat toxic, diminishing the overall vigor of the plant (V. Walbot, unpublished data; Coe et al., 1988). In *Bz2* plants cyanidin-3-glucoside is transferred into the vacuole; full pigment color develops within the vacuole by acidification of anthocyanins as well as by additional covalent modifications such as malonation (Harborne and Self, 1987; Walbot et al., 1994).

We have recently reported that the *Bz2* gene encodes a GST responsible for tagging cyanidin-3-glucoside with the tripeptide GSH, allowing it to be recognized for transport into the vacuole (Marrs et al., 1995). This transport is inhibited by vanadate, suggesting that transport is likely to occur via an MgATP-dependent, ABC-type GSH pump in the vacuolar membrane (Marrs et al., 1995). This pump was first described in plants for its ability to actively sequester GSH S-conjugates of herbicides in the vacuole (Martinoia et al., 1993). A similar pump functions in animal cells at the cell surface to actively excrete GSH S-conjugates of various toxins from tissues (for review, see Ishikawa, 1992). Thus, a common mechanism exists in plants for the transport and sequestration of toxic natural and xenobiotic compounds, with GSH conjugation being the modification that allows recognition and entry of compounds from various origins into the vacuoles (Ishikawa, 1992; Martinoia et al., 1993; Li et al., 1995; Marrs et al., 1995).

GSTs in plants have been most extensively studied with regard to herbicide detoxification; GSH conjugation is a major factor in herbicide tolerance in maize and other plants (Lamoureux and Rusness, 1989, 1993). Recently, it has been recognized that a number of "stress" genes of previously unidentified functions make up a plant GST subclass (for reviews, see Droog et al., 1993; Marrs, 1996). These type III GSTs are induced by numerous environmental stress conditions, including those arising from pathogen attack (Taylor et al., 1990), oxidative stress (Wingate et al., 1988; Levine et al., 1994; Tenhaken et al., 1995), and heavy metal toxicity (Czarnecka et al., 1988; Hagen et al., 1988; Wingate et al., 1988; Mauch and Dudler, 1993). The type III GSTs also play a role in the cellular response to both natural and synthetic auxins (Droog et al., 1993; Hahn and Strittmatter, 1994; Ulmasov et al., 1994, 1995; Droog, 1995; Droog et al., 1995). *Bz2* encodes a type III GST and *Bz2*

[1] This research was supported by National Institutes of Health grant no. GM32422 to V.W. K.A.M. was supported as a postdoctoral fellow of the Department of Energy/National Science Foundation/U.S. Department of Agriculture TriAgency Training Program in Plant Biology.

[2] Present address: Department of Biology, University of Indianapolis, 1400 East Hanna Avenue, Indianapolis, IN 46227.

* Corresponding author; e-mail vwalbot@leland.stanford.edu; fax 1–415–725–8221.

Abbreviations: *Adh1*, alcohol dehydrogenase-1; BMS, Black Mexican Sweet; *Bz1*, *Bronze1*; *Bz2*, *Bronze2*; *C2*, chalcone synthase; GSH, glutathione; GST, glutathione S-transferase; PC, phytochelatin; *rbcS*, small subunit of Rubisco; RT, reverse transcription.

transcript levels are induced by cold (Christie et al., 1994). *Bz2* is the only member of this expanding list of GSTs for which an endogenous substrate has been defined (Marrs et al., 1995; Marrs, 1996).

Because GSTs have been shown to accumulate under different stress conditions, our study was initiated to test the specificity of *Bz2* induction under a variety of stress conditions. We previously reported a curious feature of *Bz2* gene expression, namely that *Bz2* transcripts are inefficiently spliced in field-grown plants. Splicing of the single 78-base intron appears to be sensitive to environmental conditions; up to 40% of the transcript is unspliced in leaves (Nash et al., 1990; Nash and Walbot, 1992). We describe here that salt stress, hypoxia, and heat shock had no effect on *Bz2* expression, whereas ABA, auxin, cold, and heavy metals, particularly cadmium, induced *Bz2* expression. Treatment of maize seedlings or protoplasts with cadmium resulted in both an increase in *Bz2* RNA and the specific presence of the unspliced, intron-containing form of *Bz2* RNA. The increase in *Bz2* message levels during cadmium stress appears to result, at least in part, from activation of an alternative mRNA start site upstream of the normal start site. Whereas *Bz2* mRNA is highly induced by cadmium, *Bz2* GST activity is not. We discuss the significance of this finding with regard to the possible function of the Bz2 protein in the sequestration of heavy metals.

MATERIALS AND METHODS

Chemical or Environmental Stress Treatments

Maize (*Zea mays*) seeds from the W23 *Bz2* background were surface-sterilized with 10% commercial bleach and germinated in moist vermiculite on a 16-h light (23°C)/8-h dark (20°C) cycle for 10 d in an E-30B growth chamber (Percival, Boone, IA). These seedlings express all genes of the anthocyanin pathway and have purple leaf sheaths and leaf blade margins. Sets of 10-d-old maize seedlings (10 per treatment) were incubated in solutions of ABA (500 μM), auxin (2,4-D, 500 μM), sodium chloride (500 μM), cobalt chloride (100 μM), cadmium chloride (100 μM), and sodium arsenite (100 μM) or placed under conditions of cold stress (15°C), hypoxia (by flooding roots with water), or heat shock (42°C). Control seedlings were watered normally. All treatments lasted 3 h, after which leaves were immediately frozen in liquid nitrogen for RNA isolation. All treatments were replicated at least twice with separate batches of seedlings.

RNA Isolation and Northern Blot Analysis

Seedlings were treated as indicated above and total RNA was isolated from leaves using Tri-reagent (Tel-Tex, Friendswood, TX). Total RNA (25 μg) was electrophoresed through formaldehyde-agarose gels (Ausubel et al., 1987), transferred to Zeta-probe nylon membranes (Bio-Rad), and UV cross-linked (Stratalinker, Stratagene) according to the manufacturer's instructions. Northern blots were prehybridized and hybridized in formamide solution (Ausubel et al., 1987).

Individual blots were probed with the *Bz2* probe pSS390 (Fig. 1a), a gene-specific oligonucleotide probe for the small heat-shock protein hsp17.9 (Marrs et al., 1993), the PCR-derived fragment within the first exon of the maize GST III gene (*GST*) prepared for this study using the sequence reported by Moore et al. (1986), and the cDNA plasmids for the maize *C2* gene pC2-c46 (Wienand et al., 1986), the *Adh1* gene pB428 (Bennetzen et al., 1984), the *Bz1* gene pMBzR1 (Klein et al., 1989), and the *rbcS* gene pTN20 (a gift from Tim Nelson, Yale University, New Haven, CT). Probes were ^{32}P-labeled using the oligolabeling kit supplied by Pharmacia. Following autoradiography, blots were stripped of the probe and rehybridized with a ^{32}P-labeled probe for 18S rRNA to standardize for RNA loading.

Primer Extension Analysis

Total RNA (25 μg) isolated from control, heat-shocked, or cadmium-treated seedlings was annealed at 37°C to an oligonucleotide primer ^{32}P-labeled by polynucleotide kinase as described previously (Ausubel et al., 1987). The sequence of the 38-bp oligonucleotide primer BzPRI-X is complementary to sequences approximately 100 bp downstream of the mapped 5′ terminus of *Bz2* (+1) and is 5′-GCTGACCTCCCCGCCTAGCACACGCATGGTCCCGGCCG-3′.

This primer was extended with 40 units of avian myeloblastosis virus reverse transcriptase for 90 min at 42°C. The reverse-transcriptase extended products were separated on 5% polyacrylamide-urea gels and exposed to x-ray film for 24 h. Autoradiographs were quantified by densitometry.

Protoplast Electroporation and Stress Treatments

Maize protoplasts were prepared from BMS cell cultures and electroporated as described previously (Bodeau and Walbot, 1992). Expression of the native *Bz2* gene or of the genomic construct pBz2-6 was induced by the co-electroporation of 20 μg each of plasmids pR and pC1, which carry the maize anthocyanin pathway transcriptional regulators *R* and *C1*, respectively, to induce all of the structural genes of the anthocyanin pathway (Bodeau and Walbot, 1992). After an overnight recovery, protoplasts in conditioned protoplast buffer were treated with cadmium chloride (10, 50, or 100 μM), sodium arsenite (100 μM), cold (15°C), or heat (42°C), or were left untreated in conditioned protoplast buffer (control) for 2 h. Protoplasts were recovered by centrifugation and used immediately for RNA isolation. All treatments were repeated at least twice with independent batches of protoplasts.

RNase Protection Assays

Maize BMS protoplasts were electroporated with 20 μg each of the *R* and *C1* plasmids, allowed to recover overnight, and treated with different concentrations of cadmium for 2 h. Total RNA was isolated from protoplasts and incubated with a ^{32}P-labeled antisense RNA probe spanning the *Bz2* intron with flanking exon sequences (pSS390, Fig. 1) (Nash et al., 1990). Single-stranded RNA was digested with RNase A and T1 using the RPA II kit from

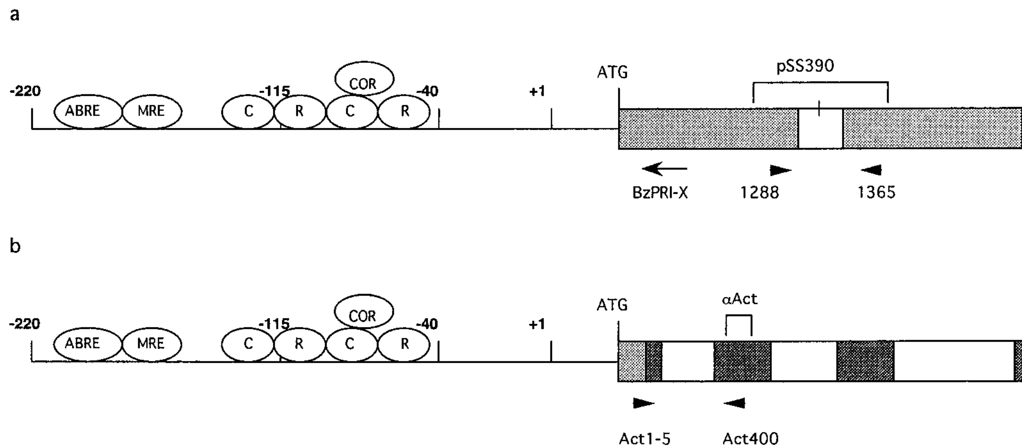

Figure 1. Schematic diagram of the constructs used in this study. a, The pBz2-6 plasmid, containing the *Bz2* gene, promoter, and regulatory sequences. Numbering is based on previous work (Nash et al., 1990; Bodeau and Walbot, 1992, 1996). The two exons are indicated as stippled boxes, and the single 78-bp intron is an open box. The primary transcript initiation site is labeled +1 (Nash et al., 1990); three other transcript initiation sites at −40, −115, and −220 (this study) are indicated as bold-faced numbers. The probe pSS390 used in this study is designated by the brackets; it spans the 78-bp intron and will hybridize to both exons. The arrow represents the region from which the primer extension probe was made. The solid arrowheads represent the locations of the RT-PCR primers. The stop codon in the intron is indicated by the small vertical line in the intron. In the upstream region, motifs involved in regulation by the maize anthocyanin pathway transcriptional regulators *R* and *C1* are indicated as ovals. The *C1* motifs in the promoter have the sequence TAACtG/CaGTTA, and the *R* motifs have the sequence CAgTG. Ovals also designate putative cold regulatory (COR) elements (CCGAC) at −105 and −117, a metal regulatory element (MRE; TGCTCTCGT) at −165, and an ABA regulatory element (ABRE; CACGTG) at −185. b, The Bz:Act fusion construct. This plasmid has the promoter, leader, and first nine nucleotides of *Bz2* (light stippled box) ligated in-frame to the actin genomic coding sequence (dark stippled boxes; introns are represented by open boxes) from the plasmid pMAc1 starting at nucleotide 16 of actin. The solid arrowheads represent the locations of the RT-PCR primers. The probe αAct used in this study for RNase protection analysis is indicated by the brackets. The plasmids are not drawn to scale.

Ambion (Austin, TX), and regions of the probe protected by complementary RNA in the sample were resolved on 5% polyacrylamide-urea gels. As a control for RNA loading, a 240-bp antisense RNA probe, αAct, complementary to maize actin (Shah et al., 1983), was also included in each reaction (Fig. 1b). A 1-bp sequencing ladder was used as a size marker.

Construction of the Bz2:Actin Fusion Construct and Expression in Protoplasts

The *Bz2* genomic clone pBz2-6 (Nash et al., 1990) was linearized with *Eag*I at the 5′ end of the coding region. The *Eag*I site was filled in with the Klenow fragment of DNA polymerase (Pharmacia), leaving a blunt end that contained the first 9 bp (ATGACGGCC) of *Bz2*. The plasmid was then digested with *Bam*HI at the 3′ end to remove all other *Bz2*-coding sequences from the plasmid. The actin clone pMAc1 (Shah et al., 1983) was digested with *Eco*RV at the 5′ end and *Bam*HI and the 3′ end of the coding region, resulting in a genomic actin fragment that contained all but the first 15 bp of actin. These two fragments were ligated to create a plasmid, pBz:Act, containing the *Bz2* promoter, leader sequences, and the first 9 bp of *Bz2* ligated in-frame to the 2189-bp actin-coding sequence starting at nucleotide 16 (Fig. 1b). A large-scale preparation of pBz:Act was obtained using the Wizard Maxiprep kit (Pharmacia).

RT-PCR Analysis of *Bz2*, the Bz2:Actin Fusion Construct, and *hsp82* in Protoplasts

RT-PCR was used to analyze expression levels of pBz2-6 and pBz:Act electroporated into protoplasts. Primers were chosen so that evidence of splicing would be apparent in the size of the PCR products. Primers 1288 and 1365 (Fig. 1a) span the single 78-bp intron of pBz2 and amplify a 142-bp fragment from the spliced transcript and a 220-bp fragment from the unspliced transcript. Primers Act1-5 and Act400 (Fig. 1b) span the first intron of pBzAct (84 bp) and amplify a 316-bp fragment from the spliced transcript and a 400-bp fragment from the unspliced transcript. The sequences of the primers are: 1288 (forward primer), 5′-GCAGCCTGCTCGTCCCGGA; 1365 (reverse primer), 5′-GCGCGGGTGTCTTCCGCGG; Act1-5 (forward primer), 5′-ATGACGGCCATCCAG; and Act400 (reverse primer), 5′-GACAGCTCTTGGCGC.

It should be noted that the Act1-5 forward primer spans the "fusion junction" that created pBz:Act; the first nine nucleotides are from the original *Bz2* gene and the next six nucleotides are from actin. This provided not only additional confirmation that the pBz:Act vector was properly constructed but also allowed us a way to distinguish between the endogenous maize actin and the Bz:Act fusion construct (no PCR fragment could result from the endogenous maize actin gene using the Act1-5 primer because it is a composite of the *Bz2* and actin genes). As a control,

RT-PCR was also performed using primers spanning the intron of the maize *hsp82* gene to test the expression of an endogenous cadmium-inducible gene. Primers hsp82f and hsp82r span the single 140-bp intron of maize *hsp82* (Marrs et al., 1993) and amplify a 380-bp fragment from the spliced transcript and a 520-bp fragment from the unspliced transcript. The sequences of these primers are: hsp82f (forward primer), 5'-GTGCACATGGCTGGCGGTGC, and hsp82r (reverse primer), 5'-GGACTTGGTCATGCCCACGCC.

RT-PCR was performed using 100 ng of total RNA, following the manufacturer's instructions for the rTth reverse transcriptase kit (Perkin-Elmer). ^{32}P-labeled dCTP was included in the reactions to allow the products to be visualized by autoradiography after agarose gel electrophoresis. Electroporations and PCR reactions were repeated twice with RNA from independent electroporations.

GST Enzyme Assay

To test the effect of cadmium on GST activity in protoplasts, protoplasts were prepared as described above and electroporated with 20 μg each of *R* and *C1* plus 2, 10, or 25 μg of pBz2-6. After an overnight recovery, protoplasts in conditioned buffer were untreated (control) or treated with cadmium chloride (50 μM) for 2 h. Protoplasts were centrifuged and used immediately for GST assays.

To test the contribution of *Bz2* to total GST activity in seedlings, isogenic *Bz2* and *bz2* stocks in both the A188 and W23 inbred lines were used. Total GST was also measured in two additional inbred lines, B73 and B37, both of which are *Bz2*. Seeds were surface-sterilized and grown in Petri dishes for 8 d in pure water or in water with 1, 10, or 100 μM of cadmium chloride. These concentrations of cadmium had no impact on the percentage of germination; however, both the root and shoot lengths were adversely affected by continuous exposure to 100 μM of cadmium.

GST activity was assayed as described previously (Mannervik and Guthenberg, 1981) using the model substrate 1 mM 1-chloro-2,4-dinitrobenzene. Briefly, 5 μL of cleared protein extracts from plants or protoplasts was combined with 1 mM GSH (Sigma) and 1 mM 1-chloro-2,4-dinitrobenzene (Sigma) in 0.1 M sodium phosphate (pH 6.5) and analyzed on a microplate reader (Molecular Dynamics, Sunnyvale, CA) to measure the rate of change in A_{340} ($\Delta A_{340} \times 10^{-3}$ min^{-1} or mA min^{-1}) μg^{-1} protein. Background levels of spontaneous 1-choloro-2,4-dinitrobenzene conjugation were subtracted. For assays with seedlings of various genotypes, the statistical significance was tested by a two-way analysis of variance.

RESULTS

Induction of *Bz2* and Other Genes by Stress Treatments

We recently reported that the BZ2 GST shares considerable amino acid sequence homology with other type III plant GSTs and that members of this GST subclass are regulated by numerous environmental stresses (Marrs et al., 1995; Marrs, 1996). To test whether the expression of the *Bz2* gene was similarly affected by environmental stresses, maize seedlings were treated with the plant hormones ABA or auxin, with conditions of cold stress, heat shock, or hypoxia, or with the heavy metals cobalt, cadmium, or arsenite. Northern blots were prepared from the RNA of these seedlings and examined for the expression of a number of different genes. These included representative genes from the anthocyanin pathway in maize: *C2*, *Bz1*, and *Bz2*. We also tested several genes known to be affected by specific environmental conditions—the small heat-shock protein *hsp17.9*, *Adh1*, maize *GST III*, and rbcS.

Figure 2 shows the results of this study. ABA induced mRNA accumulation for the *Bz2*, *hsp17.9*, and *Adh1* genes; auxin induced the *C2* and *GST III* genes, and *Bz2* very weakly; and cold induced *C2* and *Bz2*, as expected (Christie et al., 1994). Salt stress had a minimal effect on the expression of C2 and *GST III*. Hypoxia and heat shock induced only *Adh1* and *hsp17.9*, respectively. The heavy metals cobalt, cadmium, and arsenite decreased the expression of *C2* and *Bz1*, but cadmium and arsenite induced the *Bz2* GST as well as *GST III* and *hsp17.9*. The slight differences in mobility for the *GST III* RNA transcripts probably occurs because the probe (within the first exon of maize *GST III*) hybridizes to more than one isoform of GST; slightly different isoforms occur in RNAs from different stressed tissues (Marrs, 1996). In summary, salt stress, hypoxia, and heat shock had no effect on *Bz2* expression, whereas ABA, auxin, cold, and heavy metals, particularly cadmium, induced *Bz2* transcript accumulation.

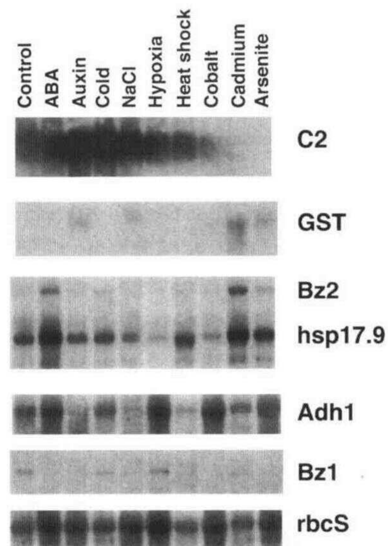

Figure 2. Expression of *Bz2* and other maize genes after various stress treatments. Ten-day-old maize seedlings were incubated in solutions of ABA (500 μM), auxin (2,4-D, 500 μM), sodium chloride (500 μM), cobalt chloride (100 μM), cadmium chloride (100 μM), or sodium arsenite (100 μM) or placed under conditions of cold (15°C), hypoxia (by flooding roots with water), or heat shock (42°C). Control seedlings were watered normally. All treatments were for 3 h, after which time RNA was isolated from seedlings. Northern blots were hybridized with DNA probes for the maize genes *Bz2*, the small heat-shock protein *hsp17.9*, GST III (GST), *C2*, *Adh1*, *Bz1*, and the *rbcS* as described in "Materials and Methods." Equivalent loading of each lane was verified by reprobing each RNA blot with an 18S rRNA probe (data not shown).

Activation of an Alternative *Bz2* mRNA Start Site by Cadmium

We next analyzed the transcription start site of the *Bz2* gene in control plants and in plants treated with either heat shock (42°C, 3 h) or cadmium (50 μM, 3 h). We prepared a hybridization probe (Fig. 1a) that consisted of a 38-bp primer, BzPRI-X, complementary to sequences approximately 100 bp downstream of the previously mapped, major 5′ terminus of *Bz2* (+1) (Nash et al., 1990). The end-labeled primer was hybridized to RNA from control, heat-shocked, or cadmium-treated seedlings and reverse transcribed with avian myeloblastosis virus reverse transcriptase. The sizes of the extended fragments were analyzed on polyacrylamide-urea gels (Fig. 3). Each RNA sample contained extended fragments corresponding to the previously determined mRNA start site of *Bz2* (+1). In addition, each RNA sample contained two other reverse-transcribed products corresponding to −40 and −115, relative to the +1 mRNA start site. These two additional 5′ termini have been noted previously as constituting about 25% of the total mRNA in protoplasts and leaves of seedlings and adult plants (Nash et al., 1990; Bodeau and Walbot, 1996). We detected an additional extended product, corresponding to −220 relative to the +1 mRNA start site but only in RNA from cadmium-stressed seedlings. This product is unique to the cadmium-treated sample and makes up approximately 20% of the mRNA in the cadmium-treated sample. These results indicate that cadmium acts to stimulate *Bz2* transcription from a start site that is not used under other conditions.

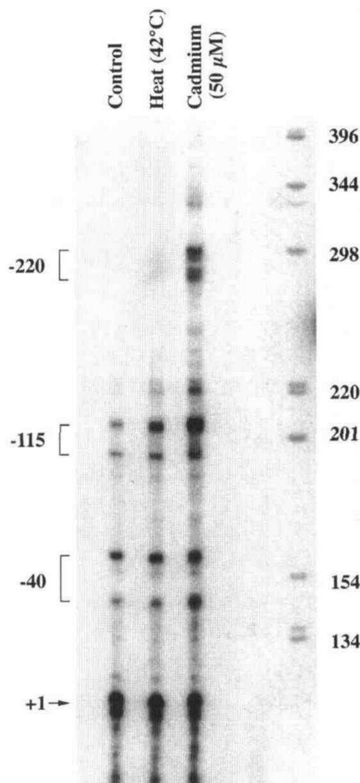

Figure 3. Primer extension analysis. Total RNA (25 μg) from control, heat-shocked, or cadmium-treated seedlings was annealed to a ^{32}P-labeled 38-bp oligonucleotide primer complementary to sequences approximately 100 bp downstream of the known 5′ terminus of *Bz2* (+1) and extended with avian myeloblastosis virus reverse transcriptase. The reverse-transcriptase extended products were separated on 5% polyacrylamide-urea gels, exposed to x-ray film for 24 h, and mapped relative to a labeled 1-kB ladder (Pharmacia). Brackets indicate a 10-bp difference in extended fragment size due to an overlap of the oligonucleotide primer within the first 100 bp of the *Bz2* gene.

Cadmium Induces *Bz2* and Increases the Amount of Unspliced Message

In previous studies we reported that splicing of the single 78-bp intron in the *Bz2* gene appears to be sensitive to environmental conditions. *Bz2* transcripts are inefficiently spliced in field-grown, but not in greenhouse-grown, plants of several different inbred lines (Nash et al., 1990; Nash and Walbot, 1992). Because the intron of *Bz2* is too small to be detected by northern blotting, we used RNase protection analysis to examine the splicing of *Bz2* under various environmental conditions. We electroporated maize BMS protoplasts with the anthocyanin pathway transcriptional regulatory genes *R* and *C1* to induce expression of the endogenous *Bz2* gene as well as the other structural genes of the anthocyanin pathway (Bodeau and Walbot, 1992). The activity of these regulatory genes has been shown to be essential for the expression of all of the structural genes of the anthocyanin pathway, and in protoplasts, they must be provided by electroporation to initiate anthocyanin biosynthesis (Bodeau and Walbot, 1992). After 16 h, protoplasts were treated with cold, heat shock, arsenite, or cadmium for 2 h. RNA was prepared from these protoplasts and used either for northern blots or RNase protection assays (Fig. 4).

As with maize seedlings, northern blots indicated that expression of *Bz2* in maize protoplasts was induced by cold and arsenite and very strongly by cadmium (Fig. 4, left). Actin levels were slightly increased by arsenite and cold and slightly decreased by cadmium, and the expression of both genes was greatly decreased by heat shock. To examine whether *Bz2* RNA splicing was also affected by cadmium, we next examined RNA levels by RNase protection analysis. RNase protection assays (Fig. 4, right), using the pSS390 probe spanning the 78-bp intron of *Bz2* (Fig. 1), demonstrated that cadmium treatment results in both a large increase in *Bz2* RNA and the presence of the protected 390-bp, unspliced, intron-containing form of *Bz2* RNA. Based on densitometric scans, expression of the protected, 312-bp, spliced *Bz2* RNA (Fig. 4, right, lower arrow) increased approximately 20-fold as a result of cadmium treatment, and the cadmium treatment increased the presence of the unspliced *Bz2* RNA (Fig. 4, right, upper arrow) more than 50-fold over the levels of unspliced *Bz2* RNA in control protoplasts. Although the antisense actin probe used in this study was not designed to span an intron (Fig. 1b), the presence of unspliced actin transcripts would be evident on the northern blots; however, we found no evidence for unspliced actin transcripts in these experiments.

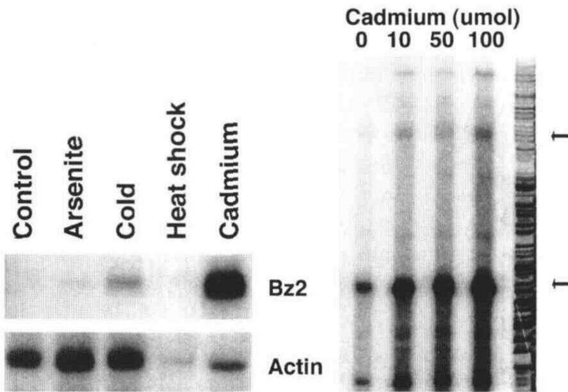

Figure 4. Cadmium induces *Bz2* and increases the amount of unspliced message. Maize BMS protoplasts were electroporated with 20 μg of the *R* and *C1* plasmids to induce expression of *Bz2*. After an overnight recovery, protoplasts were treated with cadmium chloride (10, 50, or 100 μM), sodium arsenite (100 μM), cold (15°C), or heat (42°C), or were left untreated in conditioned protoplast buffer (control) for 2 h. Total RNA was isolated from protoplasts. Northern blots (left) were probed with ^{32}P-labeled DNA probes for *Bz2* or for actin. For RNase protection (right), RNA was hybridized to the ^{32}P-labeled antisense RNA probes for *Bz2* or for actin. Single-stranded RNA was digested with RNases A and T1, and regions of the probe protected by complementary RNA in the sample were resolved on 5% polyacrylamide-urea gels as described in "Materials and Methods." Arrows indicate the location of the 312-nucleotide spliced (bottom arrow) or the 390-nucleotide unspliced (top arrow) *Bz2* mRNA; the bottom band is the protected actin mRNA (240 nucleotides). A 1-bp sequencing ladder was used as a size marker (right lane).

(The faint bands at the very top of the gel represent the wells through which the protected fragments entered the gel and do not reflect any alternative unspliced form of *Bz2*.) *Bz2* splicing does appear to be inhibited by cadmium, and thus, there are two lines of evidence that *Bz2* is transcriptionally influenced by cadmium: the appearance of a novel transcriptional start site and the accumulation of unspliced transcripts.

We attempted to induce expression of *Bz2* in protoplasts simply by treating them with cadmium alone or by electroporation of one, but not both, of the known transcriptional regulators plus cadmium. In all trials, both the *R* and *C1* transcriptional regulators were required for *Bz2* expression (data not shown).

Cadmium Does Not Inhibit Splicing of Maize Actin or *hsp82* Transcripts

We concluded from the results shown in Figures 2 and 4 that, although cadmium inhibited the splicing of *Bz2*, it did not affect the splicing of other intron-containing genes analyzed in either of these figures. Each gene examined in Figure 2 (and actin in Fig. 4) has at least one intron, the sizes of which should have been large enough to allow resolution between spliced and unspliced transcripts on the northern gel. However, we observed that for many of the transcripts, particularly *C2*, *Adh1*, *Bz1*, *rbcS*, and actin, cadmium decreased message levels, making it somewhat difficult to determine the effect of cadmium on the splicing of these genes.

To determine more directly whether the relationship between cadmium and splicing was specific to the cadmium-inducible *Bz2* or was a general effect of cadmium inducibility, we wanted to examine the expression of other cadmium-inducible, intron-containing maize genes. Two known cadmium-inducible maize genes, other than *Bz2*, are the genes for the heat-shock proteins *hsp82* and *hsp17.9*. However, these are genes that normally function under stress conditions, and we specifically wanted to examine the effect of cadmium on a gene not normally produced under stress conditions; we chose the actin gene. We made the actin gene cadmium-inducible by fusing its coding sequence in-frame to the coding sequence of *Bz2*, so that the *Bz2* promoter would drive the cadmium-inducible expression of actin (Fig. 1b, see also "Materials and Methods"). This fusion construct, pBz2:Act, contained the *Bz2* promoter, leader sequence, and first 9 bp of *Bz2* ligated in-frame to the 2189-bp actin-coding sequence starting at nucleotide 16 (Fig. 1b).

Protoplasts were electroporated with 20 μg each of plasmids pR and pC1 and either 20 μg of pBz2-6 (*Bz2* genomic plasmid) or pBz:Act (fusion construct). Protoplasts were treated with 50 μM cadmium or heat shock (42°C) or were untreated (control). The protoplasts were used immediately for RT-PCR to analyze the expression levels of pBz2-6 and pBzAct (Fig. 5). Primers were chosen so that evidence of splicing would be apparent in the size of the PCR products (Fig. 1b, see "Materials and Methods"). As an

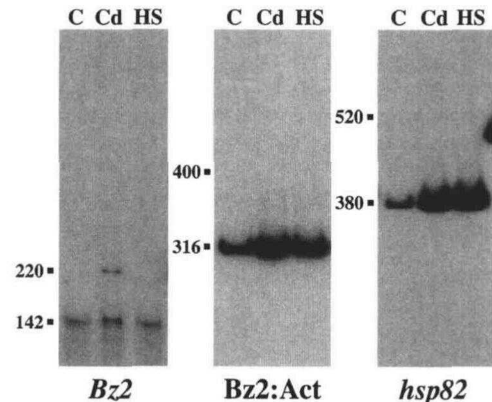

Figure 5. BMS protoplasts were electroporated with either pBZ2-6 (*Bz2* genomic clone) or pBz:Act (Bz2:Act fusion construct). Each electroporation also contained 20 μg of the *R* and *C1* plasmids to induce expression of both pBZ2-6 and pBz:Act. Splicing of three different genes was analyzed. Primers 1288 and 1365 of *Bz2* (Fig. 1a) amplified a 220-bp fragment from the unspliced message and a 142-bp fragment spliced message (left); primers Act1-5 and Act 400 (Fig. 1b) could amplify a 400-bp fragment from the unspliced message and a 316-bp fragment spliced message (middle); and, as a cadmium-inducible control (C), primers *hsp82*f and *hsp82*r could amplify transcripts from the endogenous expression of the maize *hsp82* gene (Marrs et al., 1993). A 520-bp fragment was amplified from the unspliced *hsp82* message and a 380-bp fragment spliced *hsp82* message (right). PCR products were examined by autoradiography after agarose gel electrophoresis. HS, Heat shock.

additional test, we included a set of PCR primers to detect the endogenous maize *hsp82* gene. RT-PCR analysis clearly showed the appearance of the 220-bp unspliced *Bz2* transcript as well as the 142-bp spliced transcript during cadmium treatment (Fig. 5, left). Although the actin gene was indeed cadmium-inducible when placed in the pBz2:Act fusion construct (Fig. 5, middle), the presence of the unspliced transcript was never detected. In addition, although the *hsp82* transcript was cadmium-inducible, it too showed only its fully spliced product (Fig. 5, right). Therefore, we conclude (from Figs. 2, 4, and 5) that the effect of cadmium on RNA splicing is not to act as a general splicing "poison" but, at least for the genes examined in this study, only *Bz2* appears to be specifically regulated by cadmium.

Bz2 GST Enzyme Activity in Cadmium-Treated Protoplasts and Plants

BZ2 has enzymatic activity as a GST and functions by transferring GSH to anthocyanin precursors in the cytoplasm, providing a "tag" for their transfer into the vacuole (Marrs et al., 1995). We next wanted to determine whether the increase in *Bz2* mRNA by cadmium would be reflected as an increase in the enzymatic activity of the BZ2 GST. We first examined total GST activity in BMS protoplasts electroporated with *R* and *C1* co-electroporated with increasing amounts of the *Bz2* expression plasmid pBZ2-6. As expected, GST activity increased with increasing amounts of plasmid electroporated (Fig. 6). We then compared total GST activity in protoplasts electroporated with increasing amounts of the *Bz2* plasmid and treated with 50 μM cadmium (Fig. 6). We were surprised to find that GST activity in the cadmium-treated protoplasts was no different from that in control protoplasts (Fig. 6), even though *Bz2* RNA was induced up to 20-fold higher in protoplasts after a brief treatment with cadmium (Fig. 4). These results imply that cadmium may act at somewhat opposing levels by increasing the rate of *Bz2* transcription via the use of a new transcript start site, but also by increasing the amount of unspliced *Bz2* mRNA, resulting in lower than expected increases in BZ2 GST activity.

We also surveyed various maize inbred lines to test whether GST activity in seedlings was cadmium-responsive and whether BZ2 GST activity increased in response to cadmium. Seedlings were tested for GST activity after 8 d of continuous growth in water, 1 μM cadmium, 10 mM cadmium, or 100 μM cadmium. The activity attributable to the BZ2 GST was explored by comparing GST activity in the isogenic *Bz2* and *bz2* lines of the W23 or A188 backgrounds (Table I). In addition, 10 other *Bz2* lines were also tested for total GST activity and the results from the B37 and B73 lines are shown in Table I. Results from the B37 seedlings are shown because this line had the lowest GST levels of any line examined. Data shown are from shoots, but similar patterns were observed in roots. We found that only B73 of all the lines tested showed a cadmium-responsive GST activity (Table I). In the W23 and A188 backgrounds there was no difference in GST activity between the *Bz2*- and *bz2*-containing lines. In addition, there was no difference in sensitivity to cadmium (growth rate and germination frequency) between the *Bz2*- and *bz2*-containing lines. Thus, it appears that the inbred background of the seedling is more relevant than the presence or absence of *Bz2* when examining the effect of cadmium on GST activity. More importantly, for the majority of inbred lines tested (11 of 12), cadmium does not produce an increase in GST activity.

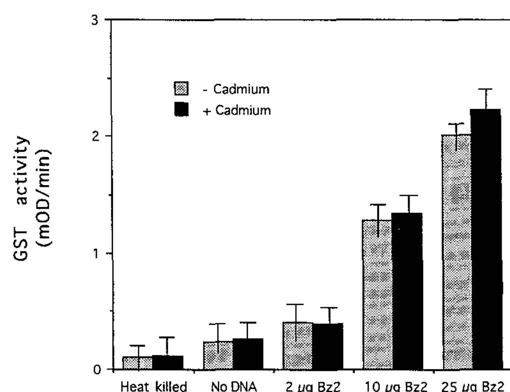

Figure 6. BMS protoplasts were electroporated with increasing amounts of the *Bz2*-expressing plasmid pBz2-6 as indicated. Expression of *Bz2* was induced by the co-electroporation of 20 μg each of the *R* and *C1* plasmids. After an overnight recovery, protoplasts were treated with cadmium chloride (50 μM, solid boxes) or in conditioned protoplast buffer alone (control, stippled boxes) for 2 h. GST activity was determined as described in "Materials and Methods." Each bar represents the average GST values from three independent electroporation experiments. Heat killed refers to the levels of GST activity found in a sample of boiled protoplast supernatant used to determine baseline GST levels. Within an experiment, all treatments were repeated twice and GST levels were determined twice for each treatment. SE bars are shown. OD, Optical density or *A*.

DISCUSSION

In this study we investigated the effect of various stress treatments on gene expression and RNA splicing of *Bz2* and other genes in maize. We show that *Bz2* is strongly and rapidly induced by cadmium and to a lesser extent by arsenite, ABA, and auxin (very weakly). Other GST genes have been shown to be induced by cadmium, including the soybean GmGST26-A gene (Czarnecka et al., 1988; Hagen et al., 1988), the tobacco NT103, NT107, and NT114 genes (van der Zaal et al., 1991; Boot et al., 1993), and the *parA* GSTs (Takahashi et al., 1991). In addition, protein levels of the wheat GST25 and GST26 increase in the presence of cadmium (Mauch and Dudler, 1993). Our study revealed that the maize *GST III* gene and the heat-shock gene *hsp17.9* are also induced by cadmium (Fig. 2). However, cadmium does not seem to be a general inducer of other stress genes (such as *Adh1*) or of other anthocyanin pathway genes (such as *C2* and *Bz1*).

In the presence of cadmium *Bz2* splicing is affected along with RNA accumulation (Figs. 4 and 5). In these respects, the response of *Bz2* is identical to that of the soybean GmGST26-A gene (Czarnecka et al., 1988; Hagen et al., 1988). Cadmium, and to a lesser extent copper and arsenite, but not zinc or heat shock, caused a 25 to 50% block in intron processing of GmGST26-A (Czarnecka et al., 1988;

Table I. *GST activity in maize seedlings exposed to cadmium*

Ten seedling shoots were harvested after 8 d of continuous exposure to the solutions indicated and tested for GST activity. GST enzyme activity was measured in triplicate from each seedling; in addition, the entire experiment was independently replicated three times. Values shown represent the averages of nine GST enzyme assays. Statistical significance was determined by two-way analysis of variance.

Inbred Line	*Bz2* Status	Total GST Activity			
		Control	Cadmium (μM)		
			1	10	100
		$\Delta A_{340}\ min^{-1}\ \mu g^{-1}$ protein			
W23	*Bz2*[a]	375	375	420	350
	bz2[a]	525	375	385	390
A188	*Bz2*[a]	500	375	420	420
	bz2[a]	410	375	420	500
B73	*Bz2*[b]	175	600	755	240
B37	*Bz2*[c]	100	90	110	130

[a] No significant differences in GST levels between *Bz2* and *bz2*. [b] GST levels at 1 and 10 μM cadmium are significantly different from the water and 100 μM values ($P < 0.05$). [c] No significant difference between treatments, but the B37 *Bz2* levels are significantly different from the other inbred lines ($P < 0.05$).

Hagen et al., 1988). The deleterious effect of cadmium, however, does not appear to reflect a widespread "poisoning" of RNA splicing. In our northern blot survey (Fig. 2), RNase protection assays (Fig. 4), and RT-PCR analysis of the Bz:Act fusion construct (Fig. 5), we saw no evidence for unspliced transcripts other than *Bz2*, although all of these test genes had one or more introns. There has been one other example in which cadmium did cause inhibition of splicing; processing of the petunia *hsp70* mRNA was inhibited during treatment with cadmium or copper but not during heat stress (Winter et al., 1988). Thus, cadmium may alter the splicing of only a limited subset of stress genes.

Bz2 transcription is also affected by cadmium (Fig. 3). The increase in *Bz2* message levels during cadmium stress appears to result, at least in part, from the use of a unique mRNA start site approximately 200 nucleotides upstream of the normal start site that is not used in unstressed or heat-stressed tissues. Again, *Bz2* is very similar to the soybean GmGST26-A in the respect that a cadmium-specific transcription start site was observed for this gene as well (Czarnecka et al., 1988). Transcripts originating from this start site may require promoter elements that are specialized to be induced in response to heavy metal toxicity. The *Bz2* promoter is known to be very complex. The regulation by the required transcriptional activators *R* and *C1* depends on numerous sequences found between the +1 transcription start site and about −115 in the *Bz2* promoter (Fig. 1, based on data from Bodeau and Walbot, 1992, 1996). There are also multiple transcription start sites, at least one of which is uniquely used during cadmium stress (Nash et al., 1990; Bodeau and Walbot, 1992, 1996; this study).

In light of our results, we examined the *Bz2* promoter region more thoroughly. We found that the *Bz2* promoter also contains several motifs common to other environmentally regulated genes: putative cold regulatory elements, a metal regulatory element, and an ABA regulatory element (Fig. 1). It is interesting that the putative metal regulatory element motif in the *Bz2* promoter (at −175) lies downstream of the cadmium-specific start site (at −220). Despite the existence of these motifs, however, activation of the *Bz2* promoter (by cadmium) still appears to act in conjunction with the *R* and *C1* transcriptional regulators, at least in BMS protoplasts. We are currently testing whether the cold regulatory element, ABA regulatory element, and metal regulatory element motifs are important for the specific responses of *Bz2* to cold stress, ABA, and cadmium, respectively.

Plants exposed to heavy metals exhibit stress responses that include induction of GSTs (Mauch and Dudler, 1993) and synthesis of PCs. PCs are Cys-rich heavy-metal-binding peptides derived from GSH with the typical structure $(\gamma\text{-Glu-Cys})_n$Gly (for reviews, see Grill et al., 1985; Rauser, 1990; Steffens, 1990; Rauser and Meuwly, 1995). Detoxification of cadmium is a multistep process. Most of the intracellular cadmium is first chelated by PCs (Grill et al., 1985), and these PC-cadmium complexes are subsequently sequestered in the vacuole (Vogeli-Lange and Wagner, 1990) via an MgATP-dependent heavy metal transporter HMT1 in the vacuolar membrane (Ortiz et al., 1995; Salt and Rauser, 1995). Detoxification requires both the production of PC-cadmium complexes and subsequent transport into the vacuole (Howden et al., 1995; Ortiz et al., 1995).

We report in this paper that cadmium activates a *Bz2* transcription start site (Fig. 3) and increases *Bz2* message levels (Figs. 2 and 4), but also specifically blocks *Bz2* RNA splicing (Figs. 4 and 5) and has no detectable effect on the enzymatic activity of BZ2 (Fig. 6). *Bz2* is known to encode a protein of 241 amino acids, with a calculated molecular mass of 26.9 kD. This protein has a clearly defined role in anthocyanin biosynthesis based on more than 50 years of genetic evidence (Coe et al., 1988; Marrs et al., 1995, and refs. therein). However, our results lead us to propose that cadmium specifically blocks *Bz2* splicing to stimulate the production of an alternative BZ2 protein during cadmium stress; this truncated protein lacks the GST activity encoded by the spliced transcript (Fig. 7). This alternative 14.1-kD protein could potentially be encoded by the unprocessed message, because there is a stop codon 14 amino acids into the *Bz2* intron that would terminate the reading

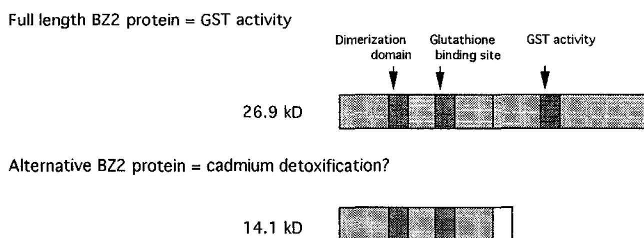

Figure 7. Model of the alternative BZ2 protein produced during cadmium stress. The *Bz2* gene has the capacity to produce two different GSH-binding proteins. The spliced *Bz2* mRNA encodes a 26.9-kD protein. An alternative 14.1-kD protein can be encoded by the unprocessed message, because the protein would terminate at the stop codon in the *Bz2* intron (open box). The first exon encodes the protein domains critical for GSH bonding and protein dimerization (dark stippled boxes); these motifs are shared by plant and animal GSTs (reviewed by Marrs, 1996). Therefore, both proteins would contain GSH-binding and protein dimerization domains, but our results indicate that only the full-length protein would have GST activity (dark stippled box).

frame (Figs. 1a and 7). The truncated BZ2 protein would contain both the proposed GSH-binding domain and protein dimerization domains, based on x-ray crystallographic analysis of other GSTs (reviewed by Marrs, 1996) but would not contain the region necessary for enzymatic activity, which is consistent with our findings (Fig. 6). The 14.1-kD alternative protein, still containing the GSH-binding site encoded within the first exon of *Bz2*, could bind the GSH moiety of a PC-cadmium complex, perhaps assisting in the sequestration of PC-cadmium complexes to the vacuole through the heavy metal transporter (HMT1) shown to be responsible for PC-cadmium transport into vacuoles (Ortiz et al., 1995).

Previous investigators have speculated that truncated proteins could be produced from the unprocessed versions of the *hsp70* and GmGST26-A messages (Czarnecka et al., 1984, 1988; Winter et al., 1988). Czarnecka et al. (1984) showed that both a 26-kD, full-length GmGST26-A protein and a 13- to 15-kD truncated protein could be in vitro translated during cadmium stress. A truncated BZ2 protein produced during cadmium stress from the unspliced *Bz2* mRNA is currently being investigated in our laboratory (J.C. Urioste, unpublished results).

It is interesting that of all maize lines examined none are naturally *bz2*. The only nonfunctional alleles of *bz2* were artificially created in genetics experiments using radiation or transposable elements (for review, see Walbot et al., 1994). Because *Bz2* is induced by many stresses and its function is preserved in maize lines, it is reasonable to hypothesize that it may have multiple roles in detoxification, with glutathionation of anthocyanin precursors being just one of the functions.

An interesting feature of this model is that the *Bz2* gene, and perhaps other type III GST genes (like GmGST26-A), has the capacity to produce two different GSH-binding proteins that serve to sequester either organic or inorganic toxins. In this model cadmium would regulate the RNA-splicing decision to produce a small, GSH-binding alternative protein that would retain the capacity to recognize GSH-containing compounds such as PC-cadmium complexes for sequestration and detoxification of cadmium in cells. Rapid production of the alternative *Bz2* product would require regulation of both transcriptional and posttranscriptional events, including activation of a novel transcriptional start site and of splicing failure. With cadmium modulating the splicing decision to give the "correct" product for detoxification of heavy metals, *Bz2* would thus be considered a metal-activated protein. Cadmium can also regulate enzymes posttranslationally; PC synthase is activated directly by cadmium (Rauser, 1995). In summary, the maize *Bz2* gene and other type III plant GSTs may encode multifunctional stress proteins. The GST activity is critical for the detoxification of natural and xenobiotic organic molecules, and the predicted alternative GSH-binding protein may be crucial for heavy metal detoxification.

NOTE ADDED IN PROOF

The GST referred to in this paper as GmGST26-A was originally termed Gmhsp26-A (Czarnecka et al., 1988) and is identical to the GH2/4 gene identified by Hagen et al., (1988). The new name GmGST26-A reflects its recent identification as a GST (Marrs, 1996).

ACKNOWLEDGMENTS

We would like to thank Gan Dunnington, Chris Sarno, Jen Taylor, and Lindsay Fletcher for performing the GST activity analysis on the maize inbred lines, and Mark Alfenito and Pepe Urioste for critical reading of this manuscript.

Received June 14, 1996; accepted October 2, 1996.
Copyright Clearance Center: 0032–0889/97/113/0093/10.

LITERATURE CITED

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K (1987) Current Protocols in Molecular Biology. Greene/Wiley Intersciences, New York

Bennetzen JL, Swanson J, Taylor WC, Freeling ME (1984) DNA insertion into intact maize tissues by electroporation. Proc Natl Acad Sci USA **81:** 4125–4128

Bodeau JP, Walbot V (1992) Regulated transcription of the maize *Bronze-2* promoter in electroporated protoplasts requires the *C1* and *R* gene products. Mol Gen Genet **233:** 379–387

Bodeau JP, Walbot V (1996) Structure and regulation of the maize *Bronze2* promoter. Plant Mol Biol (in press)

Boot KJM, van der Zaal BJ, Velterop J, Quint A, Mennes AM, Hooykaas PJJ, Libbenga KR (1993) Further characterization of expression of auxin-induced genes in tobacco (*Nicotiana tabacum*) cell suspension cultures. Plant Physiol **102:** 513–520

Christie PJ, Alfenito MR, Walbot V (1994) Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta **194:** 541–549

Coe EH, Hoisington DA, Neuffer MG (1988) The genetics of corn. *In* GF Sprague, J Dudley, eds, Corn and Corn Improvement. American Society of Agronomy, Madison, WI, pp 81–258

Czarnecka E, Edelman L, Schoffl F, Key JL (1984) Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol Biol **3:** 45–58

Czarnecka E, Nagao RT, Key JL (1988) Characterization of *Gmhsp26*, a stress gene encoding a divergent heat-shock protein of soybean: heavy-metal induced inhibition of intron processing. Mol Cell Biol **8:** 1113–1122

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD thesis. Leiden, The Netherlands
Droog FNJ, Hooykaas PJJ, Libbenga KR, van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but functionally significant homology with glutathione S-transferases and one member indeed shows *in vitro* GST activity. Plant Mol Biol **21:** 965–972
Droog FJN, Hooykaas PJJ, van der Zaal BJ (1995) 2,4-Dichlorophenoxyacetic acid and related chlorinated compounds inhibit two auxin-regulated type-III tobacco glutathione *S*-transferases. Plant Physiol **107:** 1139–1146
Grill E, Winnacker E-L, Zenk MH (1985) Phytochelatins: the principal heavy metal complexing peptides of higher plants. Science **230:** 674–676
Hagen G, Uhrhammer N, Guilfoyle TJ (1988) Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem **263:** 6442–6446
Hahn K, Strittmatter G (1994) Pathogen-defense gene *prp-1* from potato encodes an auxin-responsive glutathione-S-transferase. Eur J Biochem **226:** 619–626
Harborne JB, Self R (1987) Malonated cyanidin 3-glucosides in *Zea mays* and other grasses. Phytochemistry **26:** 2417–2418
Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7:** 1071–1083
Howden R, Andersen CR, Goldsbrough PB, Cobbett CS (1995) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol **107:** 1067–1073
Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci **17:** 463–468
Klein TM, Roth BA, Fromm ME (1989) Regulation of anthocyanin biosynthetic genes introduced into intact maize tissues by electroporation. Proc Natl Acad Sci USA **86:** 6681–6685
Lamoureux GD, Rusness DG (1989) The role of glutathione-S-transferases in pesticide metabolism, selectivity, and mode of action in plants and insects. *In* D Dolphin, R Poulson, O Arnamovie, eds, Glutathione: Chemical, Biochemical, and Medical Aspects. Wiley Interscience, New York, pp 153–196
Lamoureux GL, Rusness DG (1993) Glutathione in the metabolism and detoxification of xenobiotics in plants. *In* LJ de Kok, I Stulen, H Rennenberg, C Brunold, WE Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic, The Hague, pp 221–237
Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593
Li Z-S, Zhao Y, Rea P (1995) Magnesium adenosine 5′-triphosphate-energized transport of glutathione-S-conjugates by plant vacuolar membrane vesicles. Plant Physiol **107:** 1257–1268
Mannervik B, Guthenberg C (1981) Glutathione transferase (human placenta). Methods Enzymol **77:** 231–235
Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 127–157
Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature **375:** 397–400
Marrs KA, Silva-Casey E, Capitant SA, Bouchard RA, Dietrich PS, Mettler IJ, Sinibaldi RM (1993) Characterization of two maize HSP90 heat shock protein genes: expression during heat shock, embryogenesis, and pollen development. Dev Genet **14:** 27–41
Martinoia E, Grill E, Tommasini R, Kreuz K, Amrhein N (1993) ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. Nature **364:** 247–249
Mauch F, Dudler R (1993) Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. Plant Physiol **102:** 1193–1201
Moore RE, Davies MS, O'Connell KM, Harding EI, Wiegand RC, Tiemeier DC (1986) Cloning and expression of a cDNA encoding a maize glutathione S-transferase in *E. coli*. Nucleic Acids Res **14:** 7227–7235
Nash J, Luersen KR, Walbot V (1990) *Bronze-2* gene of maize: reconstruction of a wild-type allele and analysis of transcription and splicing. Plant Cell **2:** 1039–1049
Nash J, Walbot V (1992) *Bronze-2* gene expression and intron splicing patterns in cells and tissues of *Zea mays* L. Plant Physiol **100:** 464–471
Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem **270:** 1–8
Rauser WE (1990) Phytochelatins. Annu Rev Biochem **59:** 61–86
Rauser WE (1995) Phytochelatins and related peptides: structure, biosynthesis, and function. Plant Physiol **109:** 1141–1149
Rauser WE, Meuwly P (1995) Retention of cadmium in roots of maize seedlings. Role of complexation by phytochelatins and related thiol peptides. Plant Physiol **109:** 195–202
Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol **107:** 1293–1301
Shah DM, Hightower RC, Meagher RB (1983) Genes encoding actin in higher plants: intron positions are highly conserved but coding sequences are not. J Mol Appl Genet **2:** 111–126
Stafford H (1990) Flavonoid Metabolism. CRC, Boca Raton, FL
Steffens JC (1990) The heavy-metal binding proteins of plants. Annu Rev Plant Physiol Plant Mol Biol **41:** 553–575
Takahashi T, Kusaba M, Hiroka Y, Nagata T (1991) Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. Plant J **1:** 327–332
Taylor JL, Fritzemeier K-H, Hauser I, Kombrink E, Rohwer F, Schroder M, Strittmatter G, Hahkbrock K (1990) Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Mol Plant-Microbe Interact **3:** 72–77
Tenhaken R, Levine A, Brisson LF, Dixon RA, Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. Proc Natl Acad Sci USA **92:** 4158–4163
Ulmasov T, Hagen G, Guilfoyle T (1994) The *ocs* element in the soybean *GH2/4* promoter is activated by both active and inactive auxin and salicylic acid analogues. Plant Mol Biol **26:** 1055–1064
Ulmasov T, Liu Z-B, Hagen G, Guilfoyle TJ (1995) Composite structure of auxin response elements. Plant Cell **7:** 1611–1623
van der Zaal EJ, Droog FNJ, Boot CJM, Hensgins LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol Biol **16:** 983–998
Vogeli-Lange R, Wagner GJ (1990) Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Implication of a transport function for cadmium-binding peptides. Plant Physiol **92:** 1086–1093
Walbot V, Benito M-I, Bodeau J, Nash J (1994) Abscisic acid induces pink pigmentation in maize aleurone tissue in the absence of *Bronze-2*. Maydica **39:** 19–28
Wienand U, Weydemann U, Niesbach-Klosgen U, Peterson PA, Saedler H (1986) Molecular cloning of the c2 locus of *Zea mays*, the gene coding for chalcone synthase. Mol Gen Genet **203:** 202–207
Wingate VPM, Lawton MA, Lamb CJ (1988) Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol **87:** 206–210
Winter J, Wright R, Duck N, Gasser C, Fraley R, Shah D (1988) The inhibition of petunia hsp70 mRNA processing during $CdCl_2$ stress. Mol Gen Genet **211:** 315–319