

# A Multidrug Resistance–Associated Protein Involved in Anthocyanin Transport in *Zea mays*

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**Anthocyanin biosynthesis is one of the most thoroughly studied enzymatic pathways in biology, but little is known about the molecular mechanisms of its final stage: the transport of the anthocyanin pigment into the vacuole. We have identified a multidrug resistance–associated protein (MRP), *ZmMrp3*, that is required for this transport process in maize (*Zea mays*). *ZmMrp3* expression is controlled by the regulators of anthocyanin biosynthesis and mirrors the expression of other anthocyanin structural genes. Localization of *ZmMRP3* in vivo shows its presence in the tonoplast, the site at which anthocyanin transport occurs. Mutants generated using antisense constructs have a distinct pigmentation phenotype in the adult plant that results from a mislocalization of the pigment as well as significant reduction in anthocyanin content, with no alteration in the anthocyanin species produced. Surprisingly, mutant plants did not show a phenotype in the aleurone. This appears to reflect the presence of a second, highly homologous gene, *ZmMrp4*, that is also coregulated with the anthocyanin pathway but is expressed exclusively in aleurone tissue. This description of a plant MRP with a role in the transport of a known endogenous substrate provides a new model system for examining the biological and biochemical mechanisms involved in the MRP-mediated transport of plant secondary metabolites.**

## INTRODUCTION

Sequestration of reactive metabolites is crucial for cell survival. Plant cells in particular produce many secondary metabolites that must be excluded from the cytoplasm to prevent cellular damage. Anthocyanin pigments are a visible example of this type of plant product. Compartmentalization of anthocyanin in the vacuole is required both to limit the mutagenic and oxidative effects of synthesis pathway intermediates (Ahmed et al., 1994; Rueff et al., 1995) and for proper biological function of the final product. The biosynthesis of anthocyanin is one of the most thoroughly studied biochemical pathways in biology. Easily identified phenotypes and complete mutant viability have made anthocyanin production an ideal model for analysis of gene regulation, paramutation, and transposon biology. Recent work on the last genetically defined step in this pathway (Marrs et al., 1995; Alfenito et al., 1998) has also identified anthocyanin biosynthesis as an important model for studying the mechanisms for the detoxification and vacuolar sequestration of heterocyclic organic anions. In addition to anthocyanins, this class of compounds includes a vast array of endogenous and xenobiotic chemicals involved in pest and disease resistance, pigmentation, UV protection, intracellular and extracellular signaling, and

weed control (Abell et al., 1993; Shirley, 1996; Hammerschmidt, 1999). Understanding the molecular mechanisms involved in transporting these molecules to the correct cellular locations represents a fundamental, yet poorly understood, problem in biology.

The synthesis of anthocyanin bears a striking similarity to the detoxification of heterocyclic organic xenobiotics. Both processes occur in the cytoplasm and begin with a relatively non-reactive hydrophobic compound that is modified by a series of enzymes to introduce reactive centers and add hydrophilic molecules such as sugars or amino acids. Such modifications not only reduce hydrophobicity but can also act as tags for recognition by the proteins that transport the modified product out of the cytoplasm (Ishikawa et al., 1997). The enzymatic modifications in anthocyanin biosynthesis serve a different purpose: the alteration of the chemical properties of the molecule to fulfill its cellular functions. Anthocyanin pigment acts as UV-B sunscreen when deposited into the vacuole, a process that requires a specific glutathione S-transferase (GST) (Marrs et al., 1995). In their most well characterized role, GSTs catalyze the addition of a glutathione (GSH) molecule to a heterocyclic organic anion (Edwards and Dixon, 2000). In such instances, the GSH conjugate serves the dual purpose of increasing hydrophilicity and marking the molecule for transport by a specific family of ABC proteins, the multidrug resistance–associated proteins (MRP) (Ishikawa et al., 1997).

Also termed GS-X pumps because of their association with the transport of glutathionated compounds, the ~190-kD MRPs constitute large protein families in many eukaryotes. There are nine MRPs described in *Homo sapiens* (Dean and Allikmets, 2001), 14 in *Arabidopsis thaliana* (Sánchez-Fernández et al., 2001; Kolukisaoglu et al., 2002), and at least 12 in *Oryza sativa* (Jasinski et al., 2003). These MRPs share significant protein

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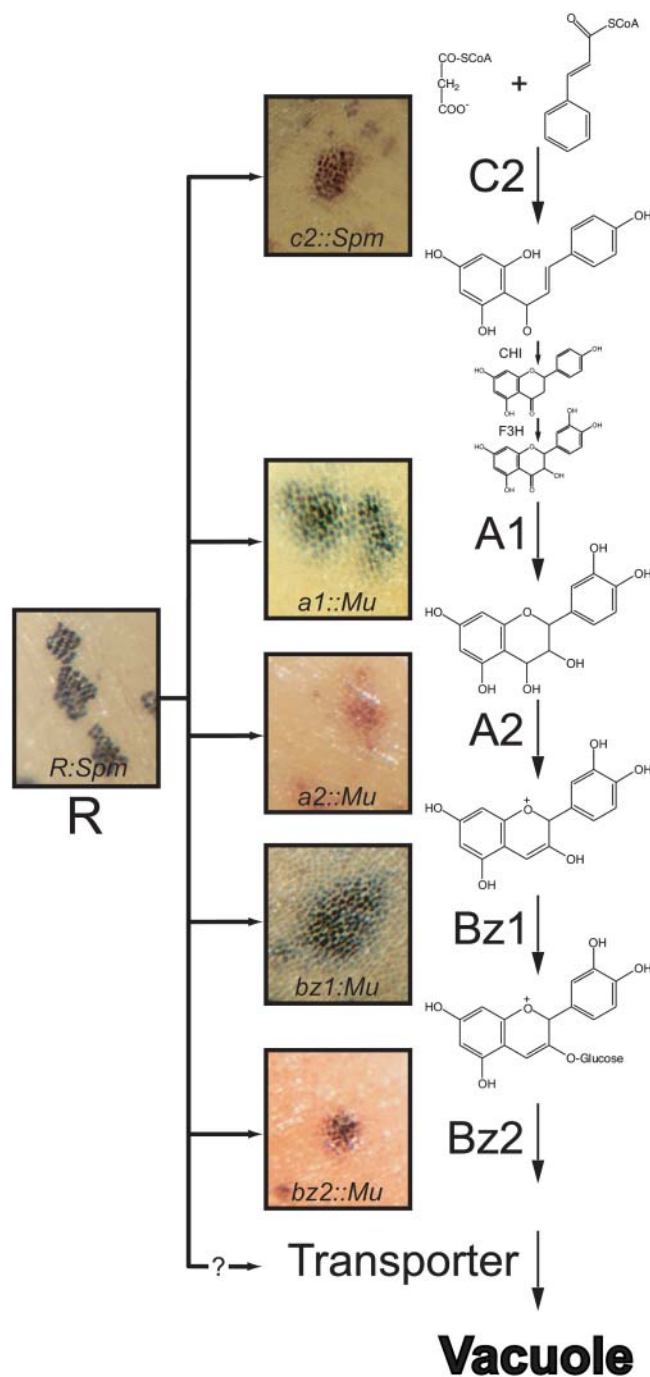
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sequence homology between species and even across kingdoms. In *A. thaliana*, 8 of the 15 MRPs are found in homologous pairs that can have >85% sequence identity (Lu et al., 1998; Kolukisaoglu et al., 2002). Interestingly, the highly homologous pair AtMRP1 and AtMRP2 shows both significant overlap of, and fundamental differences in, their in vitro substrate preferences (Lu et al., 1998; Liu et al., 2001). Therefore, both functional redundancy and unique biological roles likely exist for closely related MRPs.

Structurally, MRPs are large, modular proteins made up of three transmembrane domains (TMDs) and two ATP binding cassette domains (nucleotide binding domains [NBDs]) (Borst et al., 1999). MRPs use the energy generated by the hydrolysis of MgATP to facilitate the transmembrane movement of a variety of small molecules. Substrates are not limited to glutathionated compounds but include a range of heterocyclic organic anions and heavy metals (Rea et al., 1998). Such compounds may or may not be conjugated to sugars or amino acids and can be transported alone or in conjunction with free GSH (Liu et al., 2001). In humans, MRP substrates include anticancer drugs and other xenobiotics, including plant flavonoids, and endogenous molecules, such as bile salts and leukotrienes (Borst et al., 1999). Individual MRPs of *A. thaliana* demonstrate in vitro transport activity with herbicide metabolites, chlorophyll catabolites, and model organic substrates (Lu et al., 1997, 1998; Tommasini et al., 1998; Liu et al., 2001). Isolated plant vacuoles also actively transport chlorophyll catabolites, phytoalexins, and metabolized herbicides (Martinoia et al., 1993; Hinder et al., 1996; Klein et al., 2000). This activity is sensitive to vanadate, a specific inhibitor of ABC transporters, but is unaffected by disruptions in proton and charge gradients, indicating a specific role for ABC transporter activity (Martinoia et al., 1993). To date, however, there is no direct evidence for an in vivo substrate for any plant MRP.

Several lines of indirect evidence implicate an MRP in the anthocyanin biosynthetic pathway of *Zea mays*. Genetic analysis demonstrates that the actions of the known anthocyanin biosynthetic genes are not cell autonomous. In tissues from mutant plants, all of the anthocyanin pathway genes are present and active, with the exception of the mutated structural gene. Revertant sectors generated by unstable mutants of individual structural genes all have indistinct boundaries resulting from the sequestration of a small amount of anthocyanin in vacuoles of nonrevertant cells surrounding the revertant sector (Figure 1). This indicates that some component produced by the intact anthocyanin pathway in the revertant sector can move to the adjacent cells, partially complementing the mutation in those cells. In tissues from plants with unstable mutations in the anthocyanin regulators, in our lines a complex of the *myc* and *myb*-type proteins *B* and *Pl* in the plant body and *R* and *C1* in the aleurone, none of the anthocyanin structural genes are active outside of the revertant sectors. If the known structural genes represent all of the anthocyanin biosynthetic genes, we would expect that the components that can diffuse from structural gene revertant sectors would also be able to diffuse out of revertant sectors generated by unstable mutants of the transcription factors and that these revertant sectors would also have diffuse boundaries. However, revertant sectors of the anthocyanin transcription factors have distinct boundaries



**Figure 1.** Cell Autonomy Analysis of Anthocyanin Biosynthesis in *Z. mays*.

The anthocyanin biosynthesis pathway in maize is diagrammed accompanied by the aleurone phenotype of unstable Mutator (*Mu*) or Suppressor-mutator (*Spm*) transposon insertions in the structural genes *colorless2* (*c2::Spm*), *anthocyaninless1* (*a1::Mu*), *anthocyaninless2* (*a2::Mu*), *bronze1* (*bz1::Mu*), and *bronze2* (*bz2::Mu*) and the transcription factor *R* (*R::Spm*). Transposon excision during aleurone development produces a lineage of cells with normal gene function that form a wild-type (pigmented) sector in an otherwise mutant aleurone.

(Figure 1; Kermicle and Alleman, 1990). This cell autonomy of the anthocyanin regulatory genes argues strongly for the presence of a yet to be described structural gene that is controlled by the anthocyanin transcription factors and converts diffusible anthocyanin into a cell-limited marker. The most obvious candidate for such a gene is a membrane transporter whose expression is regulated by the anthocyanin transcription factors.

Further evidence suggests the presence of such a transporter and offers clues to its identity. The ability of vanadate, a specific inhibitor of the ABC family of transmembrane transporters, to block pigment sequestration in maize cells (Marrs et al., 1995) strongly suggests that anthocyanin transport requires the activity of an ABC transporter. Similarities between the anthocyanin biosynthesis and xenobiotic detoxification pathways, particularly the requirement for the activity of a GST to ensure proper anthocyanin localization, suggest that the ABC transporter responsible for anthocyanin sequestration is an MRP. Indeed, in a study of anthocyanin production in maize cell culture, two ABC transporters, one of which is an MRP, were identified as being upregulated in cells overexpressing the anthocyanin transcription factors *R* and *C1* (Bruce et al., 2000). In this article, we identify that MRP, *ZmMRP3*, as coregulated with the anthocyanin pathway in planta, characterize the *ZmMRP3* gene, and demonstrate the involvement of the corresponding protein in the transport of anthocyanin into the vacuole.

## RESULTS

### MRP Expression and Anthocyanin Biosynthesis

Screening of 30,000 Pioneer Hi-Bred maize EST sequences for those with homology to human, Arabidopsis, and yeast MRPs identified nine candidate MRPs. Full sequencing of the inserts yielded 1.0 to 3.0 kb (20 to 60% of estimated cDNA size) of the 3' end of seven unique genes. One of these was found to be an ABC transporter of a different class and was not characterized further. Subsequent searches of the publicly available EST database yielded 3' sequence for four additional MRP-like genes. Two of the genes identified in this screen were recently cloned and named *ZmMrp1* and *ZmMrp2* (Swarbreck et al., 2003). Fragments of the 3' end from each of these 10 genes were used as probes on RNA gel blots to compare expression patterns in eight tissue types using samples from plants with either high (*B* and *PI*) or no (*b* and *pl*) anthocyanin (Figures 2A and 2B). Six of the ten MRPs were expressed in at least one tissue (Table 1), but only *ZmMrp3* showed an expression pattern that correlated with anthocyanin levels and with the expression of *Anthocyaninless2* (*A2*), the gene encoding anthocyanidin synthase (Figure 2C). DNA gel blot analysis using the same *ZmMRP3* probe indicates that it hybridizes to a single gene (data not shown).

It was surprising to find that neither *ZmMrp3* nor *A2* were expressed at detectable levels in fully pigmented, mature adult leaves. To determine if this was a consequence of leaf age, we screened adult leaves of the same anthocyanin genotypes shortly after they emerged and before the development of full pigmentation. Both *ZmMrp3* and *A2* were highly expressed in expanding adult leaves from pigmented plants (Table 1; data not shown). This indicates that as adult leaves age and accumulate

the indelible dark pigmentation, they downregulate expression of the anthocyanin pathway genes. This downregulation appears to eliminate detectable anthocyanin gene expression by the 15th to 20th day after leaf emergence.

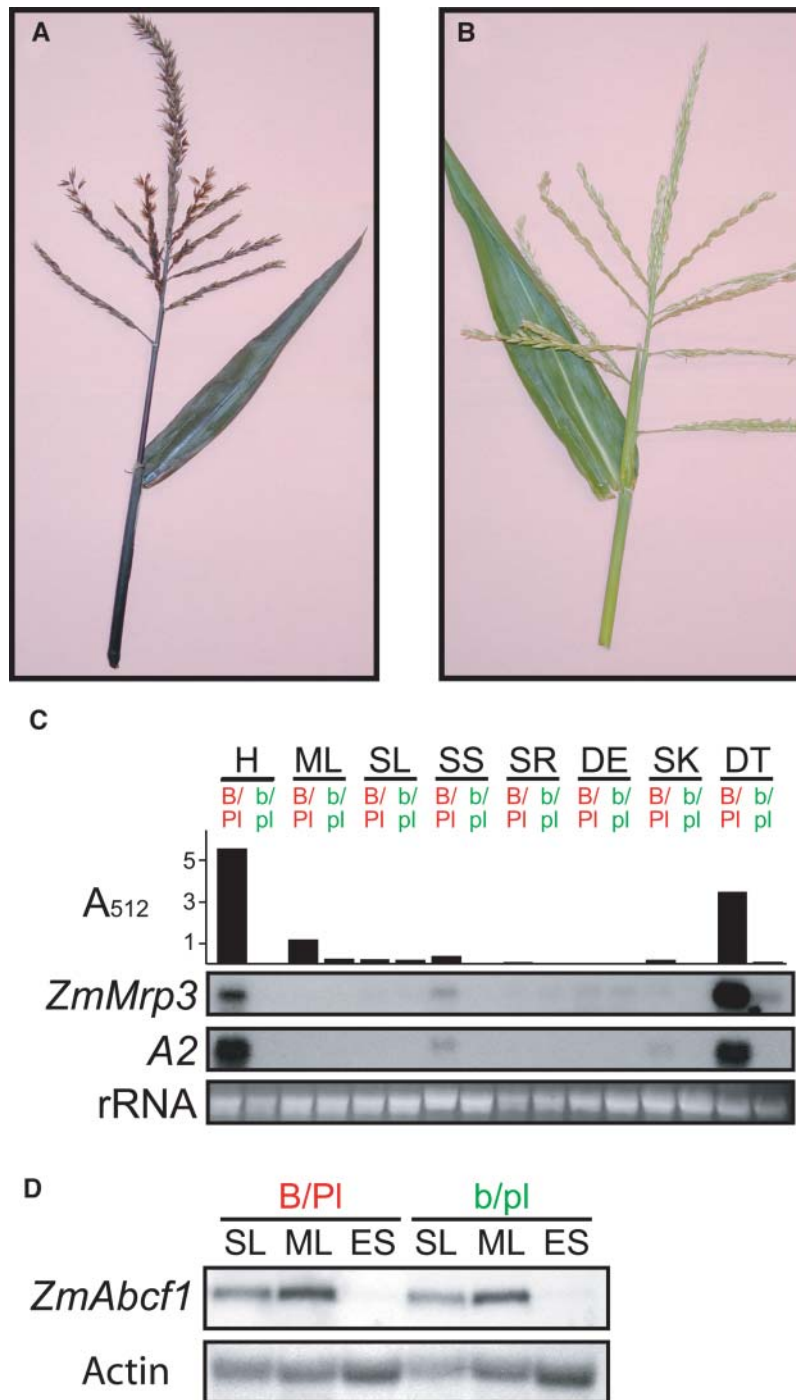
In addition to anthocyanin-regulated expression, *ZmMRP3* transcript was also found in the unpigmented developing tassel and in the developing ear, where anthocyanin is also absent (Figure 2C, Table 1). This suggests functions for this MRP in addition to anthocyanin transport. Both the developing ear and immature tassels are composed of several tissue types; therefore, the expression data offer few clues to *ZmMrp3* functions. The lack of expression of *ZmMrp3* in mature pollen and the scarcity of pollen-specific enhancer elements in the *ZmMrp3* promoter (Figure 3C) suggest that *ZmMrp3* does not have a function within pollen.

Previously, Bruce et al. (2000) reported that the expression of a second ABC protein, originally identified as an MRP, was coregulated with anthocyanin biosynthesis in Black Mexican Sweet culture cells after coexpression of the *R* and *C1* transcription factors. However, in differentiated maize tissues, the expression of this gene is regulated by light and is not affected by the expression of the anthocyanin transcription factors (Figure 2D, Table 1). Sequencing of a full-length cDNA clone of this gene allowed us to determine that it is not an MRP but is instead a member of the ABCF family of ABC proteins (Dean and Allikmets, 2001). Based on its structure, we have named this gene *ZmAbcf1*. Like the other ABCF genes described to date, the predicted ZmABCF1 protein lacks TMDs and is therefore assumed to be soluble. The other members of the ABCF family described to date are not involved in transmembrane transport. Rather, they appear to play a role in protein translation (Tysock et al., 2000).

A complete survey of the expression of the available maize MRPs (Table 1) showed that five maize MRPs are expressed in multiple tissues. Of these, *ZmMrp3*, *ZmMrp5*, *ZmMrp6*, and *ZmMrp7* are expressed in all of the tissues that synthesize anthocyanin. As noted above, however, only *ZmMrp3* shows any change in expression in response to anthocyanin content. *ZmMrp7* shows the most consistent levels of expression throughout the plant but is not expressed in silks or in mature pollen. One of the most striking findings was that none of the ZmMrps tested showed detectable expression in mature pollen.

In our RNA gel blot analysis, we found that *ZmMrp1* is expressed primarily in seedling tissues, whereas *ZmMrp2* expression is only detectable in adult leaves. Within the limits of the differing genetic backgrounds and tissue types examined in these two studies, our results for the expression of *ZmMrp1* and *ZmMrp2* agree with an RT-PCR based expression analysis reported by Swarbreck et al. (2003). The primary difference between the two studies is the detection of very low levels of *ZmMrp2* expression in all tissues tested by RT-PCR that were not shown by RNA gel blot analysis. This leaves open the possibility that genes we report as not being expressed are present but at levels below the limits of detection of RNA gel blotting.

Four of the ZmMrps are not expressed at detectable levels in the tissues tested but may be active in tissue types that were not included in this survey or, as noted above, may be present at levels below the detection limits of RNA gel blots. To shed light on



**Figure 2.** *ZmMrp3* Is Upregulated in Pigmented Tissues.

**(A)** Fully pigmented maize. Upper leaf and tassel of a maize plant with full anthocyanin pigmentation. In this maize line, anthocyanin pigmentation of the plant body is controlled by the activity of two interacting transcription factors, *B* and *Pl*.

**(B)** Maize lacking anthocyanin. Upper leaf and tassel from plants that are not expressing the anthocyanin transcription factors *B* and *Pl* and are, therefore, lacking all anthocyanin pigmentation.

**(C)** *ZmMrp3* gene expression in relation to anthocyanin synthesis. Anthocyanin content, as measured by absorbance at 512 nm, and RNA gel blot of eight maize tissues from the lines shown in **(A)** and **(B)**. Anthocyanin and total RNA were isolated from the same tissue sample. The blot was probed with the 3' UTR of *ZmMrp3* and the complete *A2* cDNA.

**(D)** *ZmAbcf1* gene expression in relation to anthocyanin synthesis. An RNA gel blot of total RNA from three maize tissues from the maize lines shown in **(A)** and **(B)**. Blots were probed with the 3' UTR of *ZmAbcf1* and with maize actin as a loading control.

H, husk; ML, mature leaf; SL, 14-d-old seedling leaf; SS, 14-d-old seedling shoot; SR, 14-d-old seedling root; DE, developing ear first day after silk emergence; SK, silks 3 d after emergence; DT, developing tassel 3 to 5 d before pollen shed; ES, 14-d-old etiolated seedling. B/Pl, tissue containing anthocyanin because of the expression of the anthocyanin transcription factors *B* and *Pl*; b/pl, tissue lacking anthocyanin because of the absence of expression of the anthocyanin transcription factors *B* and *Pl*.

**Table 1.** Relative Expression of *Z. mays* MRPs

Tissue	<i>ZmMrp</i> Designated by GenBank Accession Number																			
	1		2		3		4		5		6		7		8		9		10	
	AY186246	AY186247	AY609318	BG349642	BU050629	BU050290	BU051439	BF729302	BU098343	AW066678	B/PI	b/pl	B/PI	b/pl	B/PI	b/pl	B/PI	b/pl	B/PI	b/pl
Etiolated seedling	++	++	ND <sup>a</sup>	ND	-	-	-	-	++	++	+	+	++	++	ND	ND	ND	ND	ND	ND
Seedling root	-	-	-	-	-	-	-	-	ND	ND	ND	ND	++	++	-	-	-	-	-	-
Seedling shoot	++	++	-	-	+++	-	-	-	++	++	++	++	++	++	-	-	-	-	-	-
Seedling leaf	+	+	-	-	-	-	-	-	++	++	++	++	++	++	-	-	-	-	-	-
Developing adult leaf	-	-	ND	ND	++	-	-	-	++	++	+++	+++	++	++	ND	ND	ND	ND	ND	ND
Mature adult leaf	ND	ND	+	+	-	-	-	-	ND	ND	ND	ND	++	++	-	-	-	-	-	-
Husk	ND	ND	-	-	++++	-	-	-	ND	ND	ND	ND	++	++	-	-	-	-	-	-
Developing ear	-	-	-	-	+	+	-	-	++	++	++	++	++	++	-	-	-	-	-	-
Developing tassel	+	+	-	-	++++	++	-	-	ND	ND	ND	ND	++	++	-	-	-	-	-	-
Silk	-	-	-	-	+	-	-	-	+	+	++	++	-	-	-	-	-	-	-	-
Pollen	-	-	ND	ND	-	-	-	-	-	-	ND	ND	-	-	ND	ND	ND	ND	ND	ND

<sup>a</sup> ND, not determined.

the expression pattern of these MRPs, we examined the tissue of origin for their corresponding ESTs. Three of the *ZmMrps* were identified in tissues not included in the blot. *ZmMrp4* ESTs were recovered from libraries of stressed seedlings and *Lesion mimic* mutant leaves, *ZmMrp9* was found in a tassel primordium library, and *ZmMrp10* ESTs were recovered from both the immature embryo and tassel primordia cDNA collections. The *ZmMrp8* ESTs were recovered from a library derived from a mixture of adult tassel, silk, kernel, root, and leaf RNA, making it impossible to determine if this MRP is present at levels below the limit of RNA gel blot detection or was derived from one of the library tissues not included in our survey.

### Structure of *ZmMrp3*

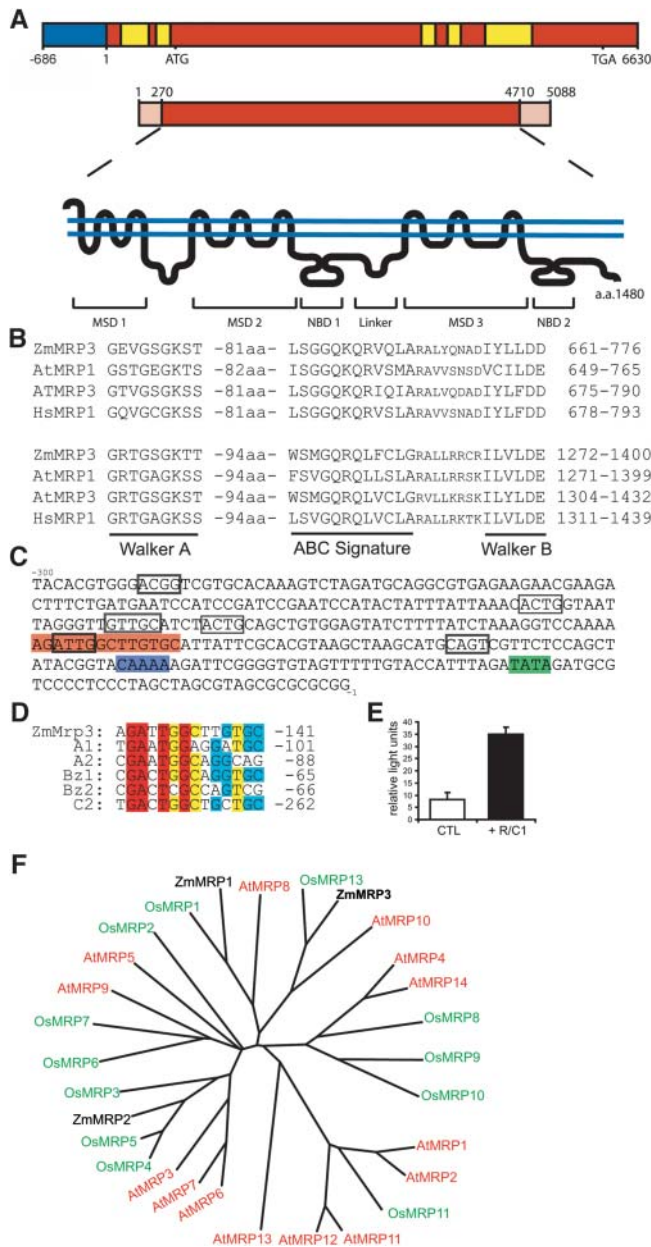
The partial clone of the anthocyanin-regulated MRP, *ZmMrp3*, contained only 1.8 kb of an estimated 5-kb cDNA; the complete transcript was recovered using rapid amplification of cDNA ends (5' RACE). A full-length cDNA was generated by RT-PCR, cloned into bacteriophage M13, and fully sequenced. The full-length *ZmMrp3* transcript is 5088 bp, with a 269-bp 5' untranslated region (UTR) and 376-bp 3' UTR bracketing a 4443-bp open reading frame encoding 1480 amino acids (Figure 3A). PCR amplification of the full-length genomic clone from inbred line A188 demonstrated a gene structure with eight exons and seven short introns (Figure 3A).

BLAST searches using the 1480-amino acid predicted protein sequence identified significant homology between *ZmMrp3* and MRPs from other organisms, particularly *A. thaliana* and *O. sativa*. BLAST CD motif searches identified two ABC TMDs and two ABC transporter ATPase motifs (NBDs) arranged in the TMD-NBD-TMD-NBD sequence characteristic of many full-sized eukaryotic ABC transporters (Theodoulou, 2000). The putative NBDs contain both the Walker A and B boxes (Walker et al., 1982)

as well as the ABC signature motif (Higgins et al., 1986) characteristic of all ABC transporters (Figure 3B). The protein sequences in these domains have extensive homology with the NBDs of *A. thaliana* MRPs 1 and 3 (Figure 3B), both of which have been shown to have ATPase and transporter activity (Lu et al., 1997; Tommasini et al., 1998). Kyte-Doolittle hydropathy analysis confirms the presence and relative location of the two predicted TMDs and also identifies a third likely TMD at the N terminus (Figure 3A). This N-terminal TMD is a unique feature of the MRP family of ABC transporters (Hipfner et al., 1997; Bakos et al., 1998), and in conjunction with the strong sequence homology with the Arabidopsis MRPs and the other structural features, identifies *ZmMRP3* as a member of the MRP family of ABC proteins.

A genomic library was screened by PCR to recover 0.69 kb of sequence upstream of the putative transcription start site. Consistent with this being a promoter sequence, the cloned region contains both TATA and CAAT motifs directly upstream of the proposed transcription start site (Figure 3C). The promoter also contains both published types of anthocyanin-responsive element (ARE) (Tuerck and Fromm, 1994; Lesnick and Chandler, 1998), which overlap (Figure 3C). Figure 3D shows the alignment of the anthocyanin-responsive element described by Tuerck and Fromm (1994) and found in all published maize anthocyanin gene promoters. Ten of the fourteen bases in the *ZmMrp3* ARE are identical to the consensus site, including all four of the bases that are conserved in this type of maize ARE (Figure 3D). Several degenerate myb-like binding (Sainz et al., 1997) sites were identified both immediately upstream and downstream from the ARE (Figure 3C). This arrangement of ARE and myb-like sites is found in the promoters of many of the maize anthocyanin structural genes and is required for proper *B/PI* and *R/C1* mediated regulation in both the *Bronze1* and *A1* genes (Roth et al., 1991; Tuerck and Fromm, 1994; Sainz et al., 1997).





**Figure 3.** Molecular Characterization of *ZmMrp3*.

**(A)** Structure of *ZmMrp3*. Diagram of *ZmMRP3* representing the known genomic sequence with promoter (blue), intron (yellow)/exon (red) structure, the cDNA sequence with open reading frame (red) and UTRs (orange), and the predicted protein structure indicating the site of putative membrane spanning domains (MSD), NBDs, and the cytoplasmic linker. a.a., amino acid.

**(B)** ABC motifs in predicted *ZmMRP3* protein. Alignment of the NBDs of *ZmMRP3*, *A. thaliana* MRP1 and MRP3, and *H. sapiens* MRP1 showing the Walker A and B boxes and the ABC signature motif.

**(C)** *ZmMrp3* promoter. Partial promoter sequence of *ZmMRP3* showing TATA (green), CAAT (blue), overlapping anthocyanin regulatory elements (ARE) (orange), and *myb*-like motifs (boxed). Note that some of the *myb* motifs may be encoded on the cDNA strand and may represent degenerate *myb* core structures that have been found to be

To further test the regulation of *ZmMrp3* by the anthocyanin transcription factors, we fused 687 bp of the *ZmMrp3* promoter, including the putative ARE site, to a luciferase reporter gene (Bodeau and Walbot, 1996) to produce the *ZmMrp3pLuc* construct. Luciferase expression from this construct was measured in maize leaf protoplasts both in the presence and absence of anthocyanin synthesis controlled by *R/C1* (Figure 3E). The expression of the reporter was regulated by the expression of the anthocyanin transcription factors, increasing approximately fourfold when present in anthocyanin producing cells.

### Sequence Relationship between *ZmMrp3* and Other MRPs

Based on alignments of the *ZmMRP3* protein sequence with sequences of the MRPs from Arabidopsis and rice, *ZmMRP3* falls into the Clade II grouping of MRPs (Kolukisaoglu et al., 2002); it is most similar to *AtMRP10* and the recently annotated rice MRP with the GenBank accession number AP005828.1, which we term *OsMRP13* (Figure 3F). It was interesting to note that the intron/exon structure of *ZmMRP3* was not entirely consistent with that found for other members of Clade II. Whereas other Clade II members have between seven and ten introns that are found exclusively in the region between the two NBDs, *ZmMRP3* has only five introns in this region but has two introns in the 5' UTR that are absent in the other members of this clade (Kolukisaoglu et al., 2002).

The most striking finding in the sequence analysis was the similarity between *ZmMRP3* and *OsMRP13*. Over the entire protein, these two genes share >79% amino acid identity. This similarity increases to 85% if the highly variable 5' TMD is excluded. When compared with the relationships between all MRPs identified from *A. thaliana* (Kolukisaoglu et al., 2002) and *O. sativa* (Jasinski et al., 2003), the level of homology between *ZmMRP3* and *OsMRP13* is found only in duplicate gene pairs from within the same species, such as *AtMRP1* and 2, 4 and 14, 7 and 8, and 12 and 13 (Kolukisaoglu et al., 2002). The closest *ZmMRP3* homolog in Arabidopsis is *AtMRP10*, which shares 60% amino acid identity with both *ZmMRP1* and *OsMRP13*. This level of homology is similar to that found among the Arabidopsis and rice MRPs that are grouped together outside of the duplicate pairs (Figure 3E). Both *OsMRP13* and *AtMRP10* were identified based on homology searches of sequenced genomes; consequently, nothing is known about their functions.

sufficient for anthocyanin promoter activation (Tuerck and Fromm, 1994).

**(D)** The *ZmMrp3* promoter contains an anthocyanin regulatory element. Alignment of the AREs, as described by Tuerck and Fromm (1994), from maize anthocyanin structural genes indicating their location relative to transcript start site.

**(E)** The *ZmMrp3* promoter is regulated by the anthocyanin transcription factors *R* and *C1*. Luciferase activity derived from the luciferase gene driven by a 687-bp fragment of the *ZmMrp3* promoter in the presence or absence of *R* and *C1*.

**(F)** *ZmMRP3* is closely related to *OsMRP13*. Unrooted phylogenetic tree comparing the full-length protein sequences of *A. thaliana* and *O. sativa* MRPs and *ZmMRP1*, *ZmMRP2*, and *ZmMRP3*.

Although the lack of full-length sequence from most of the maize MRPs precluded useful phylogenetic analysis, ClustalW comparisons of the available EST sequences did identify an extremely close homology between *ZmMrp3* and *ZmMrp4*. The alignment of the entire 1.7-kb EST sequence of *ZmMrp4* with the corresponding region of *ZmMrp3* shows a nucleotide identity of 77%, which rises to 88% if only the coding regions of the genes are compared. Comparison of the predicted protein sequence shows 90% amino acid identity over the available open reading frame. Additional genomic sequencing of *ZmMrp4* identified several differences in intron/exon structure compared with *ZmMrp3*, confirming that *ZmMrp4* is a unique gene (data not shown). As noted in Table 1, *ZmMrp4* expression was not detectable by RNA gel blot analysis in the tissues tested.

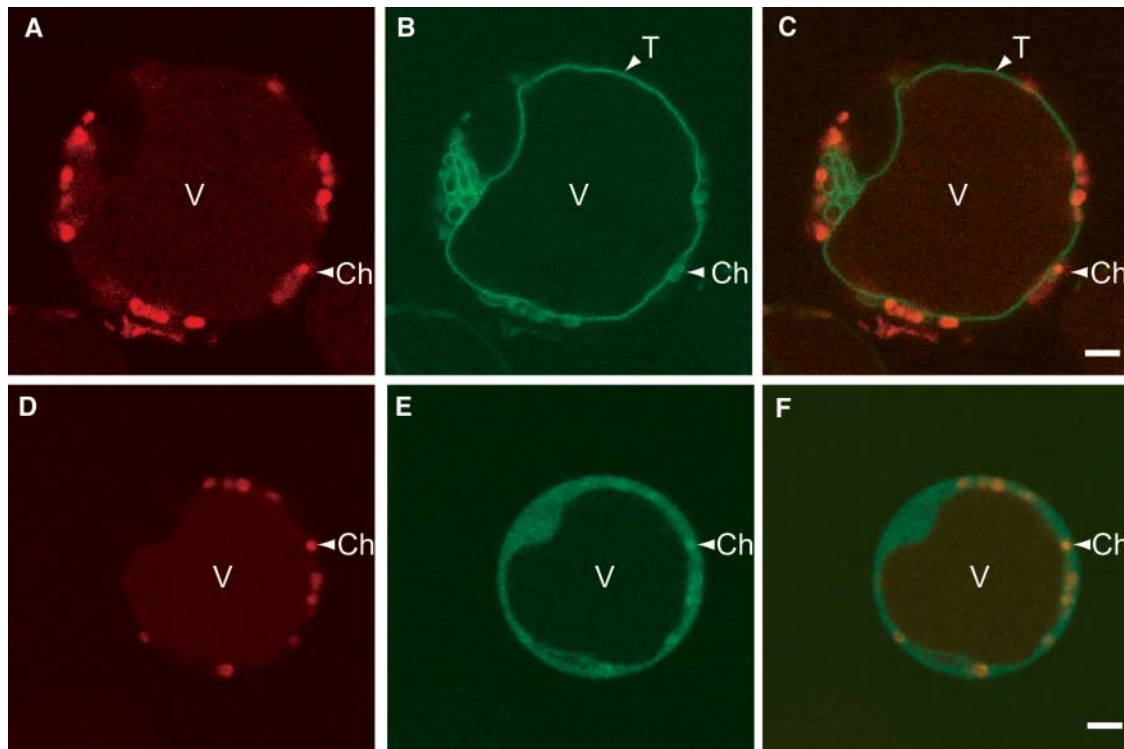
### ZmMRP3 Is Localized to the Tonoplast

To act as an anthocyanin transporter, ZmMRP3 protein must be present at the point at which transmembrane transport occurs: the tonoplast. Subcellular localization of the protein was tested using a green fluorescent protein (GFP) fusion. The vacuole was stained with LysoTracker Red (Molecular Probes), a red fluorescent dye primarily localized to the vacuole (Swanson et al., 1998).

The full-length *ZmMrp3* cDNA was fused to eGFP (Cormack et al., 1996) under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) in a fusion cassette created in bacteriophage M13. When this construct was electroporated into seedling protoplasts, fluorescence was visible only in a thin membrane outlining the large, red fluorescent central vacuole and around several smaller vacuoles (Figure 4A). Protoplasts electroporated with the empty vector showed GFP fluorescence throughout the cytoplasm (Figure 4B). It was interesting to note that protoplasts stained with LysoTracker Red showed both red and green fluorescence in the chloroplasts. Protoplasts transformed with ZmMRP3:GFP, GFP alone, and no GFP construct all showed a similar level of background green fluorescence in the chloroplast, indicating that this green fluorescence was unrelated to the expression of GFP.

### Antisense Mutants Alter Anthocyanin Biosynthesis

To test the *in vivo* role of *ZmMrp3*, we generated transgenic plants expressing an antisense construct of the 3' end of *ZmMrp3* driven by the anthocyanin-regulated promoter of the *Bronze1* gene (Figure 5A). This promoter was chosen to ensure that the antisense construct would be expressed coordinately and



**Figure 4.** Subcellular Localization of ZmMRP3.

Laser scanning confocal microscopy images of *Z. mays* leaf protoplasts. V, vacuole; Ch, chloroplast; T, tonoplast. Bars = 5  $\mu$ m.

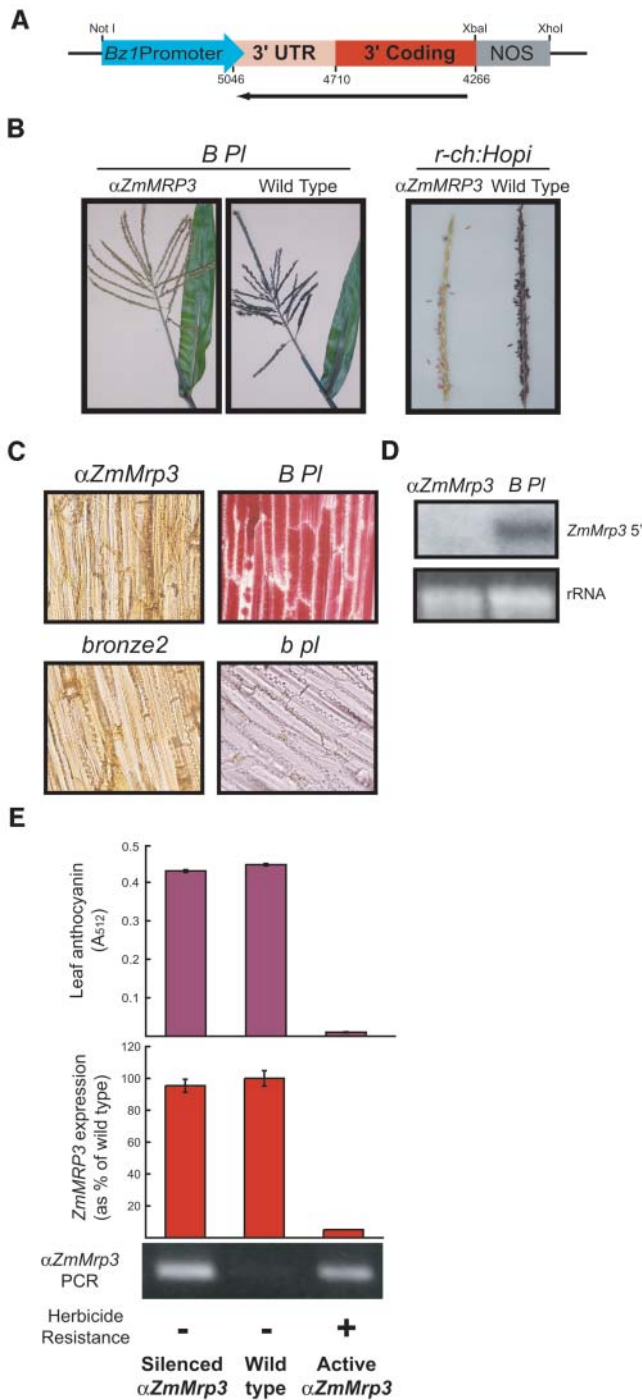
(A) to (C) Protoplasts expressing a *ZmMRP3*:GFP fusion under the control of the CaMV35S promoter.

(D) to (F) Protoplasts expressing GFP under the control of the CaMV35S promoter.

(A) and (D) Red fluorescence from LysoTracker Red staining and chloroplast autofluorescence.

(B) and (E) Green fluorescence from eGFP.

(C) and (F) Merged images showing both red and green fluorescence.



**Figure 5.** Phenotypes of Pigmented Maize Expressing the Antisense Construct  $\alpha ZmMRP3$ .

**(A)** The antisense construct  $\alpha ZmMRP3$ .  
**(B)** Phenotype of  $\alpha ZmMRP3$  expression in the adult plant. Upper leaf and tassel phenotype of sibling plants expressing ( $\alpha ZmMrp3$ ) or not expressing (wild type) the  $\alpha ZmMRP3$  transgene in the strongly pigmented *B/PI* and *r-ch:Hopi* genetic backgrounds.  
**(C)** Cellular phenotype of  $\alpha ZmMRP3$ . Microscopic images (400 $\times$  magnification) of glume face tissue taken from plants lacking anthocyanin

specifically with the anthocyanin pathway; if *ZmMrp3* performs essential roles in other cells, expression should be unaffected. Twelve independent transformants were recovered after particle bombardment. The initial transgenic lines contain nonfunctional alleles of the required anthocyanin transcription factors. Consequently, each independent transformant was introgressed into two different stocks in which deep pigmentation is expected in leaves, tassel, anthers, and/or aleurone. In one of the twelve independent transformants screened, plants carrying the transgene and the transcription factors necessary for full pigmentation showed a marked reduction in pigmentation. The observed phenotype varied with genetic background, ranging from a burnished color to an almost complete absence of pigment in anthocyanin containing tissues of the adult plant, particularly the leaves and tassel (Figure 5B). These phenotypes bear a striking resemblance to that seen in the leaves and tassel of plants carrying a mutation in the *Bz2* gene (Neuffer et al., 1997).

To define the cellular nature of this phenotype, we examined the localization of anthocyanin in the face of the glume, a single layer tissue in which pigmentation is regulated by *B/PI* expression. Consistent with this regulation, glumes from plants without anthocyanin (*b/pl*) lacked pigmentation, whereas glumes from plants with high levels of anthocyanin expression (*B/PI*) showed high anthocyanin content with the pigment localized to the vacuole (Figure 5C). Glume tissue from the  $\alpha ZmMRP3$  plants showed the bronze color characteristic of anthocyanin that cannot enter the vacuole (Marrs et al., 1995). This phenotype is strikingly similar to that seen in glumes from *bronze2* mutant plants (Figure 5C). Because of the predominance of the vacuole in glume cells, it is impossible to determine if the mislocalized anthocyanin is accumulating in the thin layer of cytoplasm or is being deposited in the cell wall.

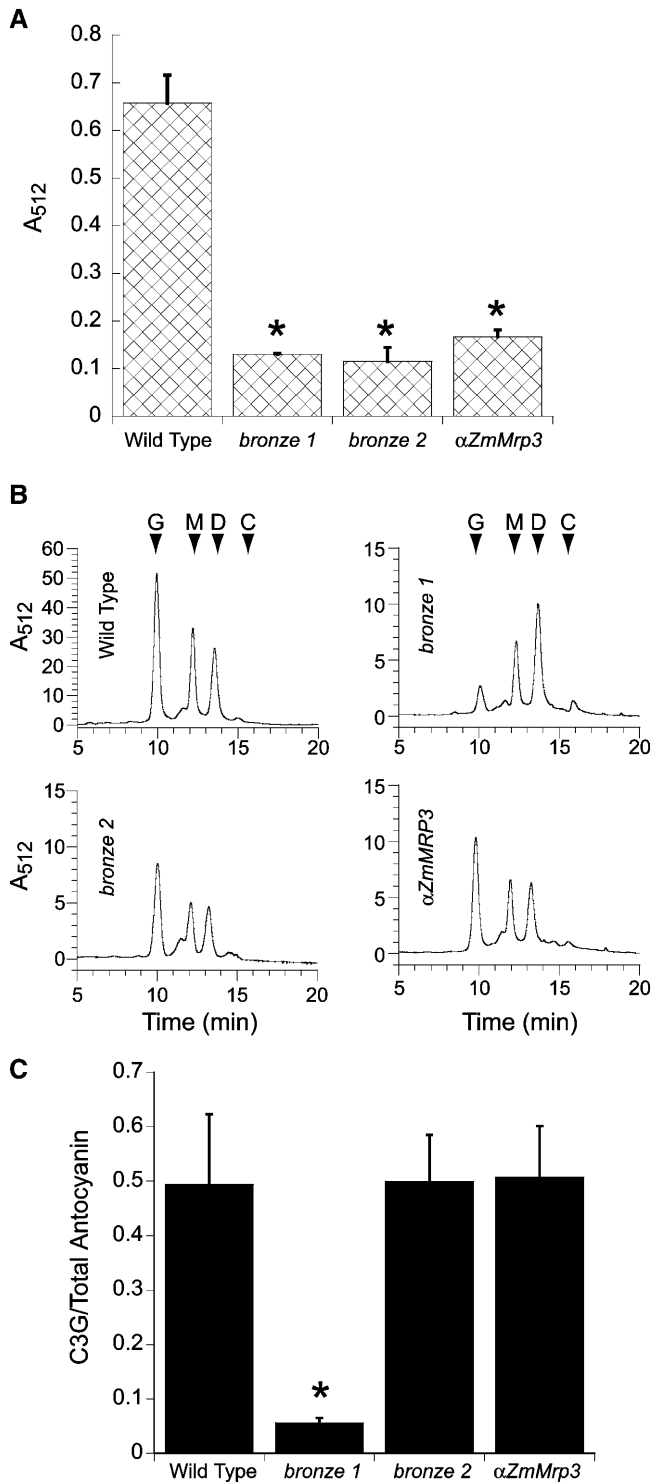
In all cases, reduced pigmentation was correlated with the presence of the transgene and elimination of the full-length *ZmMrp3* transcript as tested by RNA gel blot analysis (Figure 5D). To confirm that reduction in *ZmMRP3* expression resulted from antisense suppression and was not a product of the insertion of the transgene into an anthocyanin-related gene, we took advantage of the tight genetic linkage between the herbicide resistance marker and the transgene (data not shown) and the natural tendency of transgenes to be transcriptionally silenced over several generations of backcrossing. Silenced plants were identified by the absence of herbicide resistance combined with the

(*b/pl*), with full anthocyanin content (*B/PI*), or in a *B/PI* genetic background, but showing pigment defects resulting from the presence of either the  $\alpha ZmMRP3$  construct or the *bronze2* mutation.

**(D)**  $\alpha ZmMRP3$  eliminates the expression of *ZmMrp3*. RNA gel blot of total RNA from the leaf tissue of the *B/PI* plants in (B) showing the loss of *ZmMRP3* transcript in the antisense plants. Blots were probed with the 5' UTR of *ZmMrp3*. Ethidium bromide-stained rRNA is included as a loading control.

**(E)**  $\alpha ZmMRP3$  expression is required to produce the anthocyanin phenotype. Plants with silenced transgenes were identified by the absence of herbicide resistance combined with the presence of transgene, as shown by genomic PCR. Levels of native *ZmMrp3* expression and anthocyanin pigmentation are fully restored to wild-type levels in the presence of silenced  $\alpha ZmMrp3$ .





**Figure 6.** Antisense Plants Have Reduced Overall Anthocyanin Content but No Change in the Ratio of Anthocyanin Species Present.

**(A)** Anthocyanin content of the adult leaves of plants in *B/PI* genetic backgrounds. Shown are levels of anthocyanin in plants expressing the  $\alpha ZmMRP3$  construct and sibling lacking the transgene as well as plants with both the *bronze1* and *bronze2* mutations. Results are the means of

presence of the transgene (Figure 5E). This indicates silencing of the entire transgene array, including both the herbicide resistance gene and the antisense construct. In silenced plants, the levels of both native *ZmMrp3* expression and anthocyanin content were restored to wild-type levels (Figure 5E), confirming that transgene expression is required to produce the observed phenotype.

To characterize the biochemical nature of the color phenotype in comparison to mutants in both synthesis and localization, flavonoids were isolated from the adult leaves of sibling plants in families in which the transgene was segregating; siblings expressing the transgene are referred to as  $\alpha ZmMrp3$  plants, whereas those not carrying the transgene are referred to as wild-type plants. We also extracted flavonoids from both *bronze1* and *bronze2* mutants. The *bronze1* mutant lacks the glucosyl transferase required to synthesize cyanidin 3-glucoside from cyanidin (Larson and Coe, 1977), and *bronze2* mutants lack an anthocyanin-specific GST and, as a result, are unable to localize cyanidin 3-glucoside to the vacuole (Marrs et al., 1995). Quantitatively, the  $\alpha ZmMRP3$ , *bronze1*, and *bronze2* mutant plants tested all had a similar reduction in anthocyanin pigments compared with the purple control plants (Figure 6A). Expression levels of the anthocyanin biosynthetic genes *A1* and *A2* remain unchanged in the *bronze1*, *bronze2*, and  $\alpha ZmMrp3$  plants, whereas these genes are not expressed in *b/pl* plants, which have no detectable anthocyanin (data not shown).

HPLC analysis of the flavonoids in maize shows that the pigment profile in the *bronze1* mutant differs from that seen in the  $\alpha ZmMRP3$  and *bronze2* plants in terms of anthocyanin species present. Purple maize contains three main anthocyanin pigments: cyanidin 3-glucoside (C3G), cyanidin 3-malonyl glucoside, and cyanidin 3-dimalonyl glucoside as well as a small amount of the C3G precursor cyanidin (Figure 6B; Fossen et al., 2001). The absence of the cyanidin specific UDP:glucosyl transferase activity in the *bronze1* mutant (Larson and Coe, 1977) is reflected in the almost complete absence of C3G in *bronze1* mutants as compared with wild-type plants (Figures 6B and 6C). By contrast, anthocyanins produced by the  $\alpha ZmMrp3$  and *bronze2* plants are found in ratios indistinguishable from wild-type plants (Figures 6B and 6C), indicating that all of the enzymatic steps of anthocyanin biosynthesis remain fully functional in both the  $\alpha ZmMRP3$  and *bronze2* plants. Therefore, the reduction in anthocyanin content in the  $\alpha ZmMRP3$  and *bronze2*

three separate extractions from the same tissue type. Bars represent standard error.

**(B)**  $\alpha ZmMRP3$  plants have the same anthocyanin profile as the wild type and *bronze2* mutant plants. Reverse phase HPLC analysis of anthocyanin pigment extracted from leaf tissue described in **(A)**. Retention times of commercial anthocyanin standards are indicated by arrows.

**(C)**  $\alpha ZmMRP3$  plants have the same ratio of anthocyanin pigments as the wild type and *bronze2* mutant plants. Ratio of cyanidin 3-glucoside to total anthocyanin content in adult leaves as measured by reverse phase HPLC. Results for each background represent the mean of anthocyanin extractions from three individual plants. Bars represent standard error. G, cyanidin 3-glucoside; M, cyanidin 3-(6"-malonyl)glucoside; D, cyanidin 3-(3",6"-dimalonyl)glucoside; C, cyanidin. The asterisk indicates statistically significant difference from the wild type at  $P < 0.01$ .

plants appears to stem from the inability of C3G to be transported into the vacuole.

### The Lack of an Aleurone Phenotype Suggests Redundancy in Anthocyanin Transport

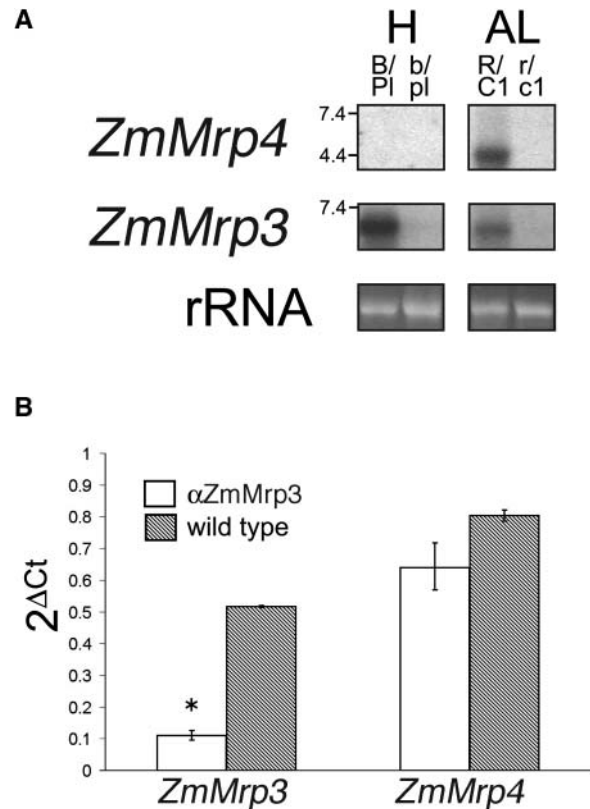
Observations of kernels from >75 ears segregating for *ZmMrp3* antisense expression showed no visible change in aleurone pigmentation. This was true of all plants, including homozygous antisense kernels derived from self-pollination of parents with a strong antisense induced phenotype in the plant body. The absence of an aleurone pigmentation phenotype suggests the presence of an aleurone-specific transporter that is able to compensate for the reduction in *ZmMrp3* expression.

To investigate this possibility, we used the suite of MRP-specific probes described earlier to screen blots containing aleurone RNA. As expected, *ZmMrp3* was expressed strongly in pigmented aleurone but was not expressed in near-isogenic unpigmented tissue (Figure 7A). This confirms that *ZmMrp3* is coregulated with the anthocyanin structural genes in the aleurone. Surprisingly, *ZmMrp4*, which is not expressed in the plant body (Table 1), is highly expressed in pigmented aleurone (Figure 7A). *ZmMrp4* was the only other MRP to show anthocyanin-regulated activity in the aleurone. It was curious to note that the *ZmMRP4* transcript at ~4.4 kb was significantly shorter than *ZmMrp3*. The extensive homology between *ZmMrp3* and *ZmMrp4* would suggest that these two genes have significant overlap in their substrate specificity, but the smaller size of the transcript indicates a large deletion in the *ZmMrp4* gene. The predicted open reading frames for *ZmMRP2*, *AtMRP13*, and *OsMRP1* are also significantly shorter than the other MRPs and hydropathy plots, and sequence alignments of the predicted proteins indicate that all three MRPs lack the 5' TMD (Sánchez-Fernández et al., 2001; Jasinski et al., 2003; Swarbreck et al., 2003). The lack of a 5' TMD domain has also been shown in human *Mrp5*, a fully active transporter (Bakos et al., 1998). Therefore, it seems likely that the 5' TMD is also absent in *ZmMrp4*. Confirmation of this through the cloning and sequencing of a full-length *ZmMrp4* gene is ongoing.

Given the extensive sequence similarity between *ZmMrp3* and *ZmMrp4*, there was a significant possibility that the  $\alpha$ *ZmMrp3* construct could suppress the expression of both genes. To test the effect that the presence of the  $\alpha$ *ZmMrp3* transgene has on *ZmMrp4* expression, we use quantitative RT-PCR to measure the expression of *ZmMrp3* and *ZmMrp4* in individual aleurones taken from an ear that was segregating for transgene expression. In aleurones that expressed the transgene, there is a fivefold reduction in *ZmMrp3* expression (Figure 7B). However, there is no statistically significant change in *ZmMrp4* expression in the presence of the transgene (Figure 7B).

## DISCUSSION

The MRPs are the most thoroughly studied of the plant ABC transporters, with many in vitro studies detailing the transport kinetics and offering suggestions of possible biological roles. This characterization of *ZmMrp3* is however the first to describe



**Figure 7.** *ZmMrp3* and *ZmMrp4* Are Expressed in the Aleurone under the Control of the Anthocyanin Transcription Factors *R* and *C1*, but Only *ZmMrp3* Expression Is Reduced by Antisense Expression.

**(A)** *ZmMRP3* and *ZmMRP4* expression is regulated by *R* and *C1* in the aleurone. RNA gel blot of total RNA from the husk and aleurone tissue of the maize lines shown in Figure 2. The same blot was probed with the 3' UTRs of *ZmMRP3* and *ZmMRP4*. Ethidium bromide-stained rRNA is included as a loading control.

**(B)** *ZmMrp4* expression is not affected by  $\alpha$ *ZmMRP3*. Quantitative RT-PCR of total RNA extracted from individual pigmented aleurones taken from three ears segregating for  $\alpha$ *ZmMRP3* expression. The presence of the transgene was detected in 8 of 12 aleurones tested. *ZmMRP3* and *ZmMRP4* expression reported as  $2^{-\Delta C_t}$  values, which represents the cycle number at which log phase amplification begins, normalized for the expression of actin (see Methods). All reactions were done in triplicate. H, husk; A, aleurone; B/PI, tissue containing anthocyanin because of the expression of *B/PI*; b/pl, tissues lacking anthocyanin because of the absence of *B/PI* expression; R/C1, tissue containing anthocyanin because of the expression of *R/C1*; r/c1, tissues lacking anthocyanin because of the absence of *R/C1* expression. The asterisk indicates statistically significant difference at  $P < 0.01$ .

the role of a plant MRP in the in vivo transport of a specific endogenous substrate. Our work demonstrates that *ZmMrp3* is strongly expressed in tissues actively synthesizing anthocyanin (Figure 2C), and its promoter is regulated by the maize anthocyanin transcription factors (Figure 3E). As well as being expressed in the expected tissue types, we also show that the *ZmMRP3* protein is localized to the tonoplast, the site of transport of the anthocyanin pigment into the vacuole (Figure 4). Transgenic

antisense plants in which *ZmMrp3* expression is reduced below detectable levels are defective in anthocyanin accumulation, but in the presence of a silenced transgene, expression and pigmentation are restored (Figures 5 and 6). Together, these data confirm the role the encoded protein plays in the transport of anthocyanin.

Although *ZmMrp3* plays a role in the proper sequestration of anthocyanin, it does not appear to be the only transporter involved in this process. The antisense knockout of *ZmMrp3* produces a strong phenotype in the leaves and tassels of pigmented plants, even when it is present as a single transgene array. However, this same construct fails to produce a phenotype in the aleurone when one, two, or three copies of the transgene array are present. In addition, the large number of genetic screens done by our lab and others have failed to identify a transporter gene as a component of the anthocyanin pathway in maize aleurone. This suggests the presence of a redundant transporter and/or an alternative and essential biological role for *ZmMrp3*.

Expression evidence argues strongly for the presence of an alternative transporter in the aleurone. *ZmMrp4*, a close homolog of *ZmMrp3*, is expressed in pigmented aleurone in a manner consistent with regulation by *R* and *C1* (Figure 7A) and is unaffected by the expression of the antisense transgene (Figure 7B). This pattern of regulation is consistent with *ZmMrp4* playing a role in anthocyanin sequestration in the aleurone and with genetic evidence suggesting that the transport step confers cell autonomy to the anthocyanin pathway. It is interesting to note that the transcript size of *ZmMrp4* is significantly shorter than *ZmMrp3*, possibly reflecting the absence of the 5'-most TMD as seen in MRPs identified in maize (Swarbreck et al., 2003) and in many other organisms (Bakos et al., 1998; Kolukisaoglu et al., 2002; Jasinski et al., 2003). Studies to confirm the structure of this *ZmMrp4* and to test its ability to transport anthocyanin are in progress.

Redundancy in anthocyanin structural genes is not uncommon. Both the chalcone synthase and chalcone isomerase genes are present in multiple copies in maize (Coe et al., 1981; Grotewold and Peterson, 1994). The chalcone synthase genes *Colorless2* (*C2*) and *White pollen* (*Whp*) present the most striking parallels to the anthocyanin transporters *ZmMrp3* and *ZmMrp4*. *C2* is expressed in all tissues that produce anthocyanin, whereas the second copy of chalcone synthase, *Whp*, is expressed in only a single tissue—the tapetum of the developing tassel (Coe et al., 1981). Either *C2* or *Whp* is sufficient to ensure flavonol synthesis in the tapetum, which is essential for the structural integrity of the pollen tube during subsequent germination (Pollak et al., 1995). This redundancy helps ensure continued male fertility in the event that one of the chalcone synthase genes is lost. *ZmMrp3* and *ZmMrp4* appear to have a similar expression pattern, with *ZmMRP3* expressed in all anthocyanin producing tissues, whereas *ZmMrp4* is expressed in a single tissue, the aleurone.

The existence of another possible mechanism of anthocyanin transport has been inferred by data from *A. thaliana*, indicating that flavonoid transport is not limited to the MRPs. The *tt12* mutant described by Debeaujon et al. (2001) yields seeds that show reduced accumulation of proanthocyanidin pigments in endothelial cells. This phenotype results from the loss of a multidrug and toxic compound extrusion (MATE) protein. MATE transporters are a recently described class of drug resistance proteins that use a Na<sup>+</sup> antiport mechanism to drive transport (Morita et al., 2000). A similar MATE-type permease has been identified as being upregulated in tomato (*Lycopersicon esculentum*) plants with high anthocyanin content resulting from overexpressing the myb-type transcription factor *Ant1* (Matthews et al., 2003), suggesting that this transport mechanism is conserved in many plant species.

The *tt12* mutant has no impact on the anthocyanin content in the Arabidopsis plant body, indicating that another transporter is

**Table 2.** Tissue-Specific Probes for RNA Gel Blots

Gene	Probe Location	Probe Construction
<i>ZmMrp1</i>	3' coding/UTR	936-bp Product from 3'RACE clone digested with <i>NcoI</i> and <i>NotI</i>
<i>ZmMrp2</i>	3' UTR	368-bp PCR product: D156F 5'-AGCCGCAATGGAATGGTTGT-3' and D157R 5'-CGTCAGG-CCTTTTAGAACAA-3'
<i>ZmMrp3</i>	3' UTR	405-bp PCR product: D63F 5'-GTGACGGGAAAGTAGTGAGTA-3' and D64R 5'-CTGCCG-CACAAGCATTCTGT-3'
<i>ZmMrp3</i>	5' UTR/coding	212-bp PCR product: D78F 5'-CATCGATCCGCGCTTTGCTTGATGGGATAA-3' and D110R 5'-GCAGCTGGAGGAGTCGAGTATGTC-3'
<i>ZmMrp4</i>	3' UTR	226-bp PCR product: DSP36F 5'-ATATCTAGAAGCCACCTC-3' and D143R 5'-TGGAAGGG-AAGCAATGAC-3'
<i>ZmMrp5</i>	3' coding/UTR	604-bp Product from EST clone digested with <i>EcoRV</i> and <i>HindIII</i>
<i>ZmMrp6</i>	3' coding/UTR	736-bp Product from EST clone digested with <i>HindIII</i>
<i>ZmMrp7</i>	3' UTR	293-bp PCR product: D58F 5'-AGTGGATCCGCACGAGTTATCCCTTGGTTCT-3' and D57R 5'-AGTGGATCCGCACGAGTTATCCCTTGGTTCT-3'
<i>ZmMrp8</i>	3' UTR	329-bp PCR product: D150F 5'-TGAAGCGGAGCAAGATCCTC-3' and D151R 5'-CGTGGA-GCCAAAGCAACCTT-3'
<i>ZmMrp9</i>	3' UTR	356-bp PCR product: D166F 5'-CTCAGATCTCGCTTTGGCATA-3' and D167R 5'-ATCTGG-GTCTGCTTCAGTATG-3'
<i>ZmMrp10</i>	3' UTR	402-bp PCR product: D168F 5'-CCCAGGTCAGCGTCAACTTGT-3' and D169R 5'-CACAC-TTCCTAGGGCGTTTGT-3'
<i>ZmAbcf1</i>	3' coding/UTR	450-bp Product from EST clone cut with <i>SstI</i> and <i>NotI</i>

involved in anthocyanin sequestration in these tissues. Indeed, maize, *Arabidopsis*, and petunia (*Petunia hybrida*) all require the activity of a specific GST for correct anthocyanin sequestration (Marrs et al., 1995; Alfenito et al., 1998; Kitamura et al., 2004). This strongly suggests a role for an MRP in this process because GST activity is intimately linked with transport by MRPs in all systems studied to date. This conclusion is further supported by the ability to generate a phenocopy of the *bronze2* mutant in maize cell lines through the application of vanadate (Marrs et al., 1995), a compound that specifically blocks ABC transporter activity but does not affect the function of MATE-type transporters (Morita et al., 2000).

The presence of a MATE-type flavonoid transporter needs to be reconciled with the GST-mediated transport of anthocyanin in maize, petunia, and *Arabidopsis*. The finding that the *tt19* gene in *Arabidopsis* encodes a GST that is required for anthocyanin pigmentation in the plant body (Kitamura et al., 2004) combined with evidence for strong upregulation of a close homolog of the anthocyanin-specific GST from petunia (Alfenito et al., 1998) in *Ant1* overexpressing tomato plants (Matthews et al., 2003) argues for the presence of both MATE and MRP transport systems in tomato and *Arabidopsis*. The simplest explanation is that these transport mechanisms are compound specific. In *bronze2* and *An9* mutants and in  $\alpha ZmMrp3$  plants, anthocyanins are mislocalized (Marrs et al., 1995; Alfenito et al., 1998; Figure 5C), whereas in *Arabidopsis tt12* mutants, the substrates in question are proanthocyanidins (Debeaujon et al., 2001). The fact that *tt12* mutants do not affect anthocyanin pigmentation argues that the TT12 transporter is not involved in anthocyanin transport in *Arabidopsis*. Interestingly, *bronze2* and  $\alpha ZmMrp3$  plants show no alteration in the localization of the flavan-4-ol phlobaphenes (data not shown) confirming the fact that structurally similar substrates can have different transport mechanisms. The upregulation of both a GST and MATE permease in tomato plants that are overexpressing the anthocyanin pathway may reflect the channeling of excess anthocyanin precursors into the synthesis of proanthocyanidins or other related compounds that utilize a MATE permease for correct localization.

Another possibility is that the MRP is not a transporter but acts to modify the activity of MATE permeases. In mammals, a subclass of the MRPs termed sulfonylurea receptors (SUR) form a multimeric complex with a potassium channel, with the SUR proteins regulating the activity of the channel proteins (Babenko et al., 1998). Whereas SUR homologs are not present in plants (Sánchez-Fernández et al., 2001), the possibility exists that an MRP, perhaps in conjunction with an anthocyanin-specific GST, interacts with and regulates the anthocyanin transport activity of a MATE permease. Indeed, the regulation of ion channels by plant MRPs has been suggested as a possible role for AtMRP5 in *Arabidopsis* (Gaedeke et al., 2001; Klein et al., 2003). Some interplay between the *Arabidopsis* MATE transporter and the TT19 GST activity is suggested by the inability of *tt19* mutant plants to efficiently sequester proanthocyanidins in the vacuole (Kitamura et al., 2004), but the development of an unusual intracellular structure as well as the inability of heterologously expressed petunia AN9 to complement only this aspect of the *tt19* phenotype raises some doubts about the involvement of an

MRP–MATE interaction. The identification of an anthocyanin-specific MRP will provide a launching point for future experiments to dissect these transport mechanisms.

Indirect evidence suggests that *ZmMrp3* may play other, possibly essential roles, in maize. The expression of *ZmMrp3* in the developing ear and unpigmented developing tassel (Figure 2C, Table 1) strongly argues for *ZmMrp3* functions beyond anthocyanin transport. This would not be surprising given the extensive range of substrates and pleiotropic mutant phenotypes found for the *A. thaliana* MRPs (Lu et al., 1998; Gaedeke et al., 2001; Liu et al., 2001), which clearly suggest multiple biological roles for this family of transporters. Further, although we were able to generate a mutant phenotype by limiting the expression of the  $\alpha ZmMrp3$  construct to tissues that accumulate anthocyanin, we were unable to recover antisense mutants driven by the constitutively expressed CaMV 35S promoter. This suggests that a loss of *ZmMrp3* function throughout the plant may negatively impact viability.

Our discovery of an anthocyanin-specific MRP provides an important model system for investigating the biology and biochemistry of both GST and MRP activity. Anthocyanin sequestration, with a known substrate and specific GST and MRP requirements, provides an excellent system for investigating several additional questions relating to the molecular mechanism of GST–MRP transport. Is the GST only involved in binding the substrate or, once bound, does it actively direct the substrate to the MRP? Does transport involve a direct GST–MRP interaction, and is this process selective? Does the GSH bound to the GST act to enhance the rate of transport? In light of the size of the GST and MRP families and the rate at which endogenous GST and MRP substrates are being identified, discovering the role of each component of this transport mechanism will be key to understanding how these molecules are efficiently moved from the cytoplasm to the correct subcellular location.

## METHODS

### Plant Material

The *R-r C1 B Pl* and *r-g c1 b pl* anthocyanin tester lines are in A188/W23 genetic background. Transgenic lines were generated in A188xB73 (Hi Type II) and crossed into the anthocyanin tester lines listed. Inbred line A188 (*r-r c1 b pl*) seedlings were grown as recommended by Sheen (1993) for protoplast isolation. Genomic and cDNA sequences were generated from RNA and DNA extracted from A188 or W23 inbred lines.

### Reagents

Cyanidin and C3G were purchased from Indofine Chemical Company (Somerville, NJ). Cyanidin 3-(6"-malonylglucoside) and cyanidin 3-(3",6"-dimalonylglucoside) were purchased from Polyphenols AS (Sandnes, Norway). LysoTracker Red DND-99 and SyberGreen were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO) except for HPLC grade solvents, which were purchased from Mallinckrodt (Paris, KY).

### Gene Expression Analysis

Total RNA was isolated, according to the manufacturer's instructions, using RNAwiz (Ambion, Austin, TX) for all tissues except aleurone.

Aleurone RNA was isolated with the Plant RNAeasy kit (Qiagen, Alameda, CA). RNA gel blotting was done as described by Raizada et al. (2001). The probes used are listed in Table 2. Probes were labeled with [<sup>32</sup>P]dCTP using the DECAprime II kit (Ambion) according to manufacturer's instructions. Probe specificity was confirmed by DNA gel blot analysis (Rudenko and Walbot, 2001).

Quantitative RT-PCR was done using an Opticon 2 (MJ Bioworks, South San Francisco, CA). Primers used for amplification were as follows: *ZmMrp3*, 5'-CATCTCTGTGGAAAGGGTTC-3' and 5'-CTGTTCGCCA-ACTATACCG-3'; *ZmMrp4*, 5'-TTCCGTGACCTCGTCAAAG-3' and 5'-TAGTTCTTCGGATGTGCAAG-3';  $\alpha$ *ZmMrp3*, 5'-CGAATAAAGCG-GACACGTT-3' and 5'-TCTAGTGAAGGCGCATCAA-3'; and actin, 5'-CGATTGAGCATGGCATTGTCA-3' and 5'-CCCACTAGCGTACAAC-GAA-3'. All primer pairs were determined to have equal amplification efficiency, and the PCR products were resolved by electrophoresis and sequenced to confirm the identity of each amplicon. The rate of amplification was monitored using SyberGreen fluorescence at 83°C. The amplification of all samples was normalized to actin amplification to account for variation in RNA levels, and all biological samples were tested in triplicate. Results are reported as  $2^{-\Delta Ct}$ , where  $\Delta Ct$  is the number of PCR cycles required to the log phase of amplification for the experimental gene minus the same measure for actin (Livak and Schmittgen, 2001), or as  $2^{-\Delta Ct}$  normalized to wild-type  $2^{-\Delta Ct}$ . Aleurone samples giving a positive signal for the  $\alpha$ *ZmMrp3* were classified as transgenic. Standard error of the mean was calculated using standard statistical methods.

#### Cloning and Characterization of *ZmMrp3* and *ZmAbcf1*

The full-length cDNA sequences of these genes were recovered using the 5' RACE system version 2.0 from Invitrogen (Carlsbad, CA) according to manufacturer's instructions with the following modification. Nested PCR was used to amplify the 5' RACE product. The product of the first PCR reaction was separated on agarose gel, and the DNA present in that section of the gel corresponding to the expected size of the 5' RACE fragment was purified. This gel-purified DNA was used as the template for the second PCR reaction.

Full-length cDNA clones were amplified by PCR using Taq HiFi (Invitrogen) with the following primers. For *ZmMrp3*: D109F, 5'-CCA-TCGATATCCGCACGCGCTTTAGCTACTA-3', and D95R, 5'-TACC-GCGGATTAGATGTGTATGACCAGTACTC-3'; for *ZmABCF1*: D88F, 5'-GCGGATCCACCAACCAACCAAGAAACAGAGG-3', and D89R, 5'-GGAATTCATAGCATACGGCACAGCAGCAGAGT-3'. *ZmMrp3* is toxic in standard *Escherichia coli* cloning strains so it was cloned into the *Clal/SstII* sites of Phagescript M13 (Stratagene, La Jolla, CA). *ZmABCF1* was cloned into the *EcoRI/BamHI* sites of pBluescript SK- (Stratagene). Both clones were fully sequenced. Genomic sequence of *ZmMrp3* was recovered by PCR with Taq HiFi. Primers designed to sequences throughout the gene were used to ensure 3 $\times$  coverage of the entire gene. Promoter sequence was recovered by digesting genomic DNA with *HindIII* and ligating it into pBluescript KS+. The resulting library was amplified by nested PCR using Taq HiFi. Amplification was done in two rounds with M13 reverse and T3 sequencing primers and the gene-specific primers D110R, 5'-GCAGCTGGAGAGTTCGAGTATGTC-3', and D129R, 5'-TCCC CGCGGATGATTCGTCTCAACTCCA-3'. The product was TA cloned into the pGEM-T vector (Promega, Madison, WI). Three independent clones were sequenced.

ARE motifs in *ZmMRP3* were identified by manual sequence analysis and using the MEME system (Bailey and Elkan, 1994). The proximal 687 bp of the *ZmMrp3* promoter were cloned into the luciferase construct described by Bodeau and Walbot (1996). The resulting construct was electroporated into maize (*Zea mays*) leaf protoplasts following the protocol of Sheen (1993) in the presence or absence of the anthocyanin transcription factor R and C1. Luciferase expression was determined as previously described (Bodeau and Walbot, 1996).

#### Phylogenetic and Sequence Analysis

ClustalW alignment of DNA and protein sequences was done with the Megalign package (DNASTar, Madison, WI). Protein distance matrix, bootstrap values (1000 replicates), and neighbor-joining consensus trees were calculated using PHYLIP (Felsenstein, 1989). *Arabidopsis thaliana* and rice (*Oryza sativa*) sequences were recovered according to Kolukisaoglu et al. (2002) and Jasinski et al. (2003). In one instance where a discrepancy was found between the annotation of *OsMRP1* and *OsMRP10* in published articles and National Center for Biotechnology Information (NCBI) databases, the NCBI designations were used.

#### GFP Constructs and Confocal Scanning Microscopy

Because the full-length clone of *ZmMrp3* was toxic to *E. coli*, a GFP construct was constructed in Phagescript M13. The CaMV 35S promoter from pMR51 (Raizada and Walbot, 2000) was cloned into the *KpnI/SalI* sites of Phagescript M13. The enhanced GFP (Cormack et al., 1996) ocs 3' cassette from pEZS-NL (D. Ehrhardt, <http://deepgreen.stanford.edu>) was cloned into the *EcoRI/SpeI* site of pZERO-2 (Invitrogen). The resulting construct was cut with *SstI* and *SstII* and cloned into the Phagescript M13 carrying the CaMV 35S promoter to produce the 35SGFPM13 vector. Full-length *ZmMrp3* was cloned into the *Clal/SacII* sites of 35SGFPM13, resulting in *ZmMrp3*:GFP. The *ZmMrp3*:GFP fusion site was sequenced to ensure that the two genes were in frame.

*ZmMrp3*:GFP was electroporated into etiolated maize protoplasts as described (Sheen, 1993) with the following modifications. The final electroporation was done in 1 mL of buffer with 50  $\mu$ g of *ZmMrp3*:GFP DNA. Protoplasts were incubated overnight in the light. Protoplasts were then incubated for 4 h in protoplast incubation solution containing 10  $\mu$ M LysoTracker Red DND-99 and washed three times in protoplast incubation solution before viewing.

Scanning confocal imaging was done using a Nikon Diaphot inverted fluorescence microscope (Tokyo, Japan) with a Nikon 60 $\times$  1.2 numerical aperture water immersion objective and a Bio-Rad MRC 1024 confocal head. Images were analyzed with LASERSHARP software (Bio-Rad, Hercules, CA).

#### Transgenic Plants

Two antisense vectors were constructed for bombardment. The *ZmMrp3* cDNA was amplified by PCR using the primers D60F 5'-GGAATTC-ATCGATCTTATCATGGCTCTTCTTCCTC-3' and D59R 5'-GGAATT-CAGATCTTAAAGCCTTGAACAGCCT-3'. To produce the *ZmMrp3* antisense construct driven by the CaMV35s promoter, this PCR product was cut with *XbaI* and *BglI* and cloned into the *BamHI/XbaI* sites of pMR51 (Raizada and Walbot, 2000) resulting in clone p35S $\alpha$ *ZmMrp3*. The *ZmMrp3* insert was fully sequenced. To create an antisense clone driven by the *Bronze1* promoter, the PCR product was cut with *XbaI* and *Clal* and cloned into pBluescript SK to create pBSKcd. This clone was fully sequenced. The promoter region from the *Bronze1* genomic clone pMbZr1 (Klein et al., 1989) was cut out with *PstI* and *BamHI* and cloned into pZERO-2. This construct was then cut with *XhoI* and *HindIII* and cloned into pBSKcd. The resultant construct was cut with *EcoRI* and *XhoI*, and the nos 3' region from pMR51 (Raizada and Walbot, 2000) was inserted, producing construct  $\alpha$ *ZmMrp3*.

Both constructs  $\alpha$ *ZmMrp1* and p35S $\alpha$ *ZmMrp1* were submitted to the Iowa State Plant Transformation Facility (Ames, IA), which produced transgenic plants by the particle bombardment method (<http://www.agron.iastated.edu/ptf/Web/-mainframe.htm>). Twenty-nine independent transformants were received, 10 expressing the p35S $\alpha$ *ZmMrp1* construct and 19 expressing the pBz1 $\alpha$ *ZmMrp3* construct, as measured by RNA gel blot analysis. PCR screening for the presence of the transgene was



done under standard conditions using the same  $\alpha ZmMrp3$  primers used in the expression analysis. Further characterization of the transgenic plants is discussed in Results.

### Pigment Extraction, Quantification, and HPLC

Anthocyanin pigments for absorbance analysis were extracted by grinding 250 mg of tissue in 1 mL of 0.1 N HCl in methanol. The ground tissue was left in the HCl/methanol overnight and then centrifuged for 5 min at full speed in a microcentrifuge to pellet the solid plant material. Absorbance of a 200- $\mu$ L volume of the supernatant was read at 512 nm using a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA).

Anthocyanin extractions for HPLC analysis were done as above except the tissue was ground in 0.1 N HCl/methanol for 1 min and immediately spun at full speed in a microcentrifuge for 5 min. The supernatant was removed and centrifuged for a further 5 min. The resulting supernatant was diluted with 5% acetic acid in ratios ranging from 1:1 to 20:1, depending on tissue type, and immediately loaded onto the HPLC.

HPLC was done using a Dionex GP40 gradient pump (Dionex, Sunnyvale, CA) using a Microsorb 100-5 C18 column (Varian, Palo Alto, CA). Data were collected and analyzed using PEAKNET software (Dionex, Sunnyvale, CA). Pigment separation was by gradient elution with a flow rate of 0.75 mL/min. Solvent A, 5% acetic acid; solvent B, acetonitrile, 1 min at 90% A, 10% B; from 90% A, 10% B to 55% A, 45% B in 17.5 min; to 100% B in 2.5 min, at 100% B for 1 min; to 90% A, 10% B in 3 min; at 90% A, 10% B for 3 min. Absorbance was detected at 512 nm using a Dionex AD20 detector.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AP005828.1, AY609318 (*ZmMrp3*), and AY615521 (*ZmAbcf1*).

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