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Functional Complementation of Anthocyanin Sequestration in the Vacuole by Widely Divergent Glutathione *S*-Transferases

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Glutathione *S*-transferases (GSTs) traditionally have been studied in plants and other organisms for their ability to detoxify chemically diverse herbicides and other toxic organic compounds. Anthocyanins are among the few endogenous substrates of plant GSTs that have been identified. The *Bronze2* (*Bz2*) gene encodes a type III GST and performs the last genetically defined step of the maize anthocyanin pigment pathway. This step is the conjugation of glutathione to cyanidin 3-glucoside (C3G). Glutathionated C3G is transported to the vacuole via a tonoplast Mg-ATP–requiring glutathione pump (GS-X pump). Genetically, the comparable step in the petunia anthocyanin pathway is controlled by the *Anthocyanin9* (*An9*) gene. *An9* was cloned by transposon tagging and found to encode a type I plant GST. *Bz2* and *An9* have evolved independently from distinct types of GSTs, but each is regulated by the conserved transcriptional activators of the anthocyanin pathway. Here, a phylogenetic analysis is presented, with special consideration given to the origin of these genes and their relaxed substrate requirements. In particle bombardment tests, *An9* and *Bz2* functionally complement both mutants. Among several other GSTs tested, only soybean *GmGST26A* (previously called *GmHsp26A* and *GH2/4*) and maize *GSTIII* were found to confer vacuolar sequestration of anthocyanin. Previously, these genes had not been associated with the anthocyanin pathway. Requirements for *An9* and *Bz2* gene function were investigated by sequencing functional and nonfunctional germinal revertants of *an9-T3529*, *bz2::Ds*, and *bz2::Mu1.*

INTRODUCTION

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) conjugate the glutathione tripeptide (γ-Glu-Cys-Gly; GSH) to a broad variety of substrates. They are an ancient and ubiquitous gene family encoding ~25- to 29-kD proteins that form both homodimers and heterodimers in vivo. GSTs are as plentiful as they are diverse. As a group, maize GSTs are among the most abundant nonphotosynthetic enzymes in plant cells, making up as much as 1% of the soluble leaf protein (Sari-Gorla et al., 1993). At least 38 plant GSTs have been identified (Marrs, 1996), and a search of the Arabidopsis expressed sequence tag (EST) database identifies >200 distinct ESTs with substantial homology to GSTs. It is striking that the GST family is so highly divergent; only a motif of ~15 amino acids is moderately conserved in sequence and position among all plant GSTs (Marrs, 1996).

Mammalian GST genes are divided into five groups (α, μ, π, σ, and θ) based on sequence similarities, immunological cross-reactivity, and substrate specificity. All identified plant GSTs fall into the most ancient class, that of theta. Droog et al. (1995) recently proposed three subdivisions of plant GSTs—type I, type II, and type III—based on a combination of sequence conservation, immunological cross-reactivity, and intron/exon structure of the gene. Type I GSTs have two introns, and many are induced, both transcriptionally and translationally, by environmental perturbations, such as dehydration (Kiyosue et al., 1993), wounding (Kim et al., 1994), active oxygen (Bartling et al., 1993), pathogen attack (Dudler et al., 1991), or the hormones auxin and ethylene (Zhou and Goldsbrough, 1993; Takahashi et al., 1995). Type II GSTs have nine introns; however, to date, the only reported sequence is from carnation (Meyer et al., 1991; Itzhaki and Woodson, 1993). Type III GSTs have a single intron and are transcriptionally and translationally inducible by various phytohormones (Takahashi et al., 1989; Droog et al., 1993), pathogen attack (Taylor et al., 1990), and heavy metals (Czarnecka et al., 1988; Hagen et al., 1988; Marrs and Walbot, 1997).

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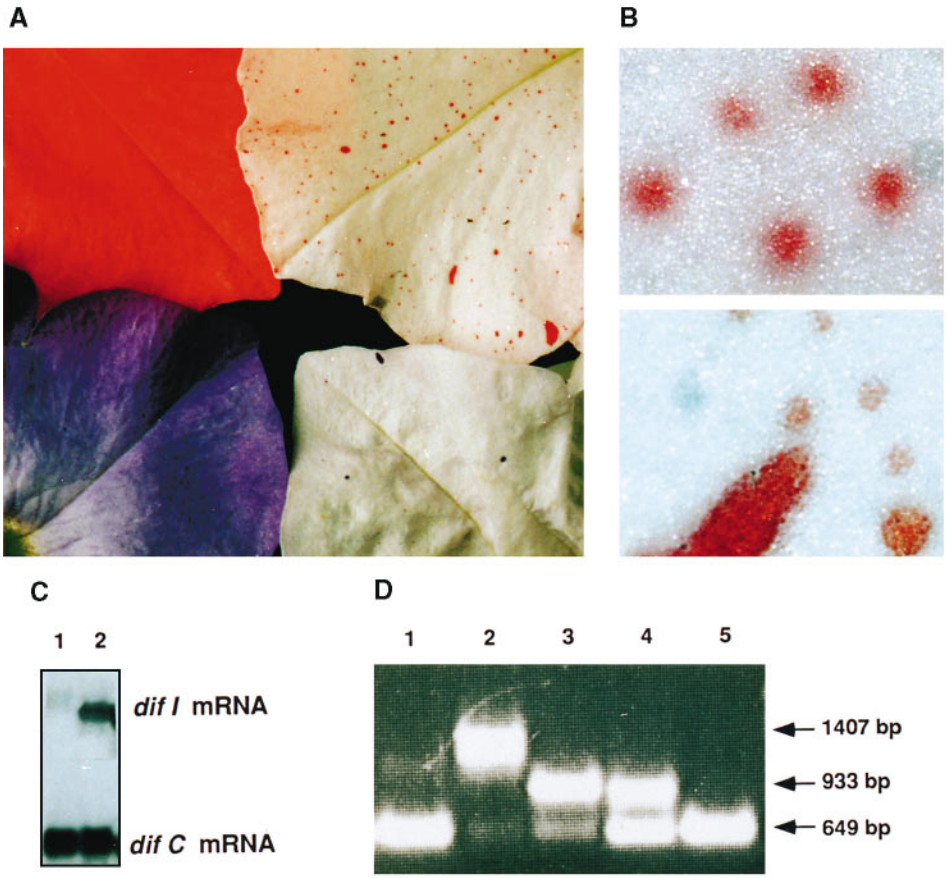

E

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dTph4 CAGGGGCGGAGCCACCTGGTGGCAAGGGGT-TCAATTGAACCCCCTTCGCCGGAAATTTGCGCGTTTTGCTTGTTTAAAGT
dTph1 ••••••••••••T••T•••••CA•••••••G•••••••••••••T••GCG•-A••••••A•A•TG•A•ATA•••GAT••AA

dTph4 TTACCAAATATCCGTAATAT-CTAAGTCTTGAACCCCCTTCAGACATGAAAAAACACTGGCGCAGATGGCAAGGGGGTTCA
dTph1 ••GGTTT•G••TG•AT••••A••GT••G••••••••••••TGA•••ATTGTTGG••T•A•TT••-•••••G•A•••••GTC

dTph4 AGATTATGCGGATGGTCCCTTGTTCGAGTCCTGG-TGATAGCAG--TTTTCCATTTTTCAGAGAGAAAGTGAATACTTTTC
dTph1 •ATGCTG•GCTCACCA•••AG•A••••A••TG••C••CC•CA•ATA••••TG•••••------------------------

dTph4 ACCAATAATTTCCATCTATTTTCCCATTCTATCATTGTGGTCCCTGGTCCCGCTAACTCTTCTCAATGCGTGCTTCTATT
dTph1 ----------------------------------------------------------------------------

dTph4 TCCTAGAACTAGAAAGGACCAAACCCCCACATAAGCTCATTTCTCTCCAATTTTGTTACCTTTACCCCTTTTTCTCTCAC
dTph1 ----------------------------------------------------------------------------

dTph4 TTTAATTTTTCCTTTTAAGTAATTACCATAATTAGGAAAGCTATCACCTATATTATATGTATTAGTAATAAACAAATGTA
dTph1 ----------------------------------------------------------------------------

dTph4 AATCGTGCTCAATCCTACCATAATTAATGATTAATATCTCAAAATCCCCTGAATAATCTAAACTTTCTAAAACTATATTT
dTph1 ----------------------------------------------------------------------------

dTph4 TTTCACCCATCTTATTCCCTCCATTTTAAAATGGTGTGGCACAATGGGTCCCAAGCGAGTTTCGGATAATGATATTGTTA
dTph1 ----------------------------------------------------------------------------

dTph4 GGCTTAATCATTAAATATTGTCTTTTAAAAAACTATGCACCACTTTATTAGAAAATTTTACTGTTCATCTATT-TCAAAT
dTph1 ----------------------------------------------CCTC•••••TAT••G•A•GG••G••CTC•

dTph4 CCTGAACCCCCTTTCTGAAATATCTCCTGCTCCGCCCCTG
dTph1 -•••••••••••••AT•A••••-•••-TG••••••••••••
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Figure 1. Transposon Tagging and Isolation of the *an9* Locus.

The principal focus of research on plant GSTs has been to define species-specific capacities to detoxify agricultural chemicals, particularly herbicides. Many classes of herbicides are glutathionated in the cytoplasm, with the conjugate being sequestered in the vacuole. Because plant species express diverse GSTs with varying substrate specificities, selectivity of herbicide action often reflects differential rates of herbicide detoxification via this glutathionation pathway. Although the role of GSTs in acute detoxification of applied chemicals has been well established, very little is known about the functions they perform in normal cellular processes. Edwards et al. (1991) reported that cinnamic acid is an endogenous substrate of GSTs in legumes. We have shown that anthocyanin (Marrs et al., 1995) and the isoflavonoid medicarpin, a legume phytoalexin (Li et al., 1997), are also endogenous GST substrates. Covalent glutathionation is a prerequisite for sequestration through a glutathione pump (GS-X pump) in the tonoplast membrane, as has been established by in vitro studies of isolated vacuoles (Martinoia et al., 1993; Li et al., 1995).

Anthocyanins are blue-red flavonoid pigments that are synthesized by most angiosperms. The initial steps of the anthocyanin pathway are highly conserved among flowering plants, whereas the terminal steps catalyze diverse ring decorations, such as acylation, sugar addition, and sulfation, yielding >2000 distinct pigments. Stable anthocyanin pigmentation occurs when the molecules are transferred to the vacuole. In the presence of a functional *Bronze2* (*Bz2*) gene, maize anthocyanins accumulate exclusively within the vacuole. When *Bz2* is missing, anthocyanins accumulate in the cytosol, conferring a tan-bronze phenotype from pigment oxidation (Marrs et al., 1995). Marrs et al. (1995) demonstrated that cyanidin 3-glucoside (C3G), the cytoplasmic product of the anthocyanin pathway, is a substrate for glutathionation by the GST encoded by *Bz2.*

We were curious to determine, based on the precedent for conservation of enzyme sequence and function in the anthocyanin biosynthetic pathway, whether a type III GST similar to *Bz2* would be involved in a late step of anthocyanin biosynthesis in another angiosperm. To explore the general requirement for a GST in anthocyanin sequestration, we cloned *An9*; this petunia gene encodes a genetically defined late step of the anthocyanin pathway. In vitro evidence demonstrates that *An9* encodes a functional GST. On the basis of sequence analysis, we determined that *An9* is a type I GST and consequently is highly divergent from the type III *Bz2* gene. Nevertheless, we demonstrate that *Bz2* and *An9* can functionally complement mutant *bz2* and *an9* tissues. This unexpected finding led us to ask whether other type I and type III GSTs not previously implicated in pigment deposition also could complement the *bz2* mutation. By functional analysis, we show that the type III soybean *GmGST26A* gene and a type I maize gene (*GSTIII*) both complement the *bz2* mutation. Other type I GSTs, including the Arabidopsis GST EST *H36860*, which is the cloned gene most closely related to petunia *An9*, plus maize *GSTI* and *GSTIV* failed to confer pigment deposition. Our results indicate that closely related GST enzymes have distinct substrate preferences and, conversely, that highly divergent enzymes can recognize the same substrate.

RESULTS

Isolation of the *An9* Locus of Petunia

In a random transposon mutagenesis experiment using the highly mutagenic petunia line W138, we identified a new mutable allele in anthocyanin biosynthesis (Figure 1A). Thin-layer chromatography analysis showed that these mutant flowers accumulated reduced amounts of the red-colored C3G expected in this background, but there was little or no effect on flavonol accumulation (data not shown). The mutant locus initially was referred to as *An13* (Quattrocchio et al., 1993; Walbot, 1996), but subsequent crosses showed it to be an allele of the uncharacterized locus *An9* (Van Houwelingen et al., 1998). Two observations indicate that

Figure 1. (continued).

(A) Phenotypes of petunia wild-type and unstable *an9* alleles. At top left is an *An9*$^+$ flower in the W138 background. At top right is an *an9* mutable flower in W138 background (*an9-T3529*). At bottom left is an *An9*$^+$ flower from Novartis Seeds breeding fields. At bottom right is an *an9* mutable flower in the same background (*an9-W2463*).

(B) Revertant spots on flowers of *an9-T3529* have diffuse boundaries (top). For comparison, cell-autonomous revertant spots on an *an1* mutable flower are shown (bottom).

(C) RNA gel blot analysis of total RNA extracted from *an9-T3529* mutant floral buds (lane 1) and wild-type floral buds (lane 2) hybridized with a *difI* cDNA probe (top) or a *difC* cDNA probe (bottom).

(D) PCR analysis of the *difI* gene in plants with the following genotypes: lane 1, *An9*$^+$/*An9*$^+$; lane 2, *an9-W2463*/*an9-W2463*; lane 3, *an9-T3529*/*an9-T3529*; lane 4, *an9-T3529*/*an9-V503-8*; lane 5, *an9-V503-9*/*an9-V503-9*. The *an9-V503-8* and *an9-V503-9* alleles are revertant and stable recessive derivatives from *an9-T3529.* Product sizes are indicated on the right.

(E) Alignment of the *dTph4* insertion found in *an9-W2463* with *dTph1* from the *Rt* locus (Kroon et al., 1994). Within the conserved transposon termini, identity is indicated by a dot; a dash indicates a mismatch throughout the alignment.

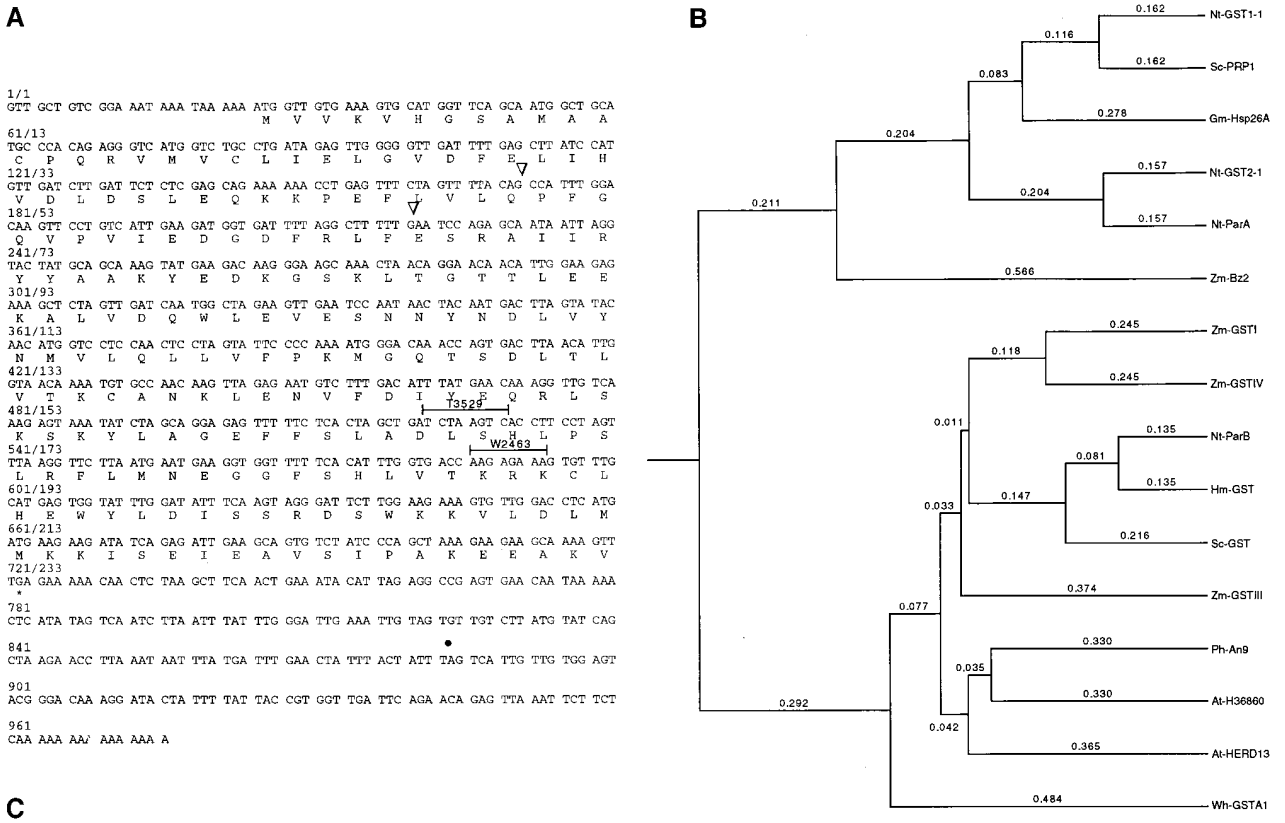

Figure 2. Nucleotide and Deduced Amino Acid Sequence of the *An9* cDNA with Amino Acid Alignment and Phylogenetic Analysis of Various Plant GSTs.

(A) The sequence of the longest cDNA is shown. The site of polyadenylation found in two other cDNAs is indicated above the sequence by a black dot. The position of the introns is indicated by open triangles. The 8-bp target site duplications created by *dTph1* in *an9-T3529* and *dTph4* in *an9-W2463* are overlined. The stop codon is marked with an asterisk.

the new mutation is caused by insertion of a transposable element. First, revertant somatic spots were present on a pale background in *an9-T3529* mutant flowers. Second, progeny derived from *an9-T3529* yielded two types of stable derivatives: plants with wild-type red flowers and plants with recessive, pale flowers. A second unstable *an9* allele (*an9-W2461*) was identified during a screen of the breeding fields of Novartis Seeds. Thin-layer chromatography analysis of *an9-W2461* flower extracts indicated that anthocyanin accumulation (malvidin rutinosides in this genetic background) was reduced, whereas flavonol accumulation was not affected. Closer inspection of the revertant spots on both *an9-T3529* and *an9-W2461* flowers showed that they had diffuse boundaries (Figure 1B), implying that *An9* controls pigmentation in a non-cell-autonomous manner. In contrast, regulatory genes such as *An1* (Quattrocchio et al., 1993) control pigmentation cell autonomously (Figure 1B). Therefore, we assumed that *An9* encodes a cytoplasmic enzyme yielding a diffusible product.

By using differential cDNA cloning, we isolated a set of cDNA clones (called *difA* to *difl*) that are transcriptionally controlled by the *An1*, *An2*, and *An11* regulatory genes of the anthocyanin biosynthetic pathway but whose function(s) was not known (Kroon et al., 1994). To determine whether one of these cDNA clones was derived from the *An9* locus, we analyzed their corresponding mRNAs in flowers with *an9-T3529* or the wild-type progenitor allele (Figure 1C). All *dif* clones hybridized equally with RNA from *an9-T3529* and wild-type corolla limbs, with the exception of *difl.* The *difl* clone hybridized with a 1-kb mRNA in wild-type but not in *an9-T3529* corolla limbs. Instead, *an9-T3529* corollas contained a low amount of a slightly larger transcript.

To determine whether the altered transcripts resulted from a rearrangement in the *difl* gene, we sequenced the ends of the *difl* cDNA and designed polymerase chain reaction (PCR) primers (see Methods). PCR analysis showed that *an9-T3529* plants harbored an insertion of $\sim$300 bp in the *difl* gene (Figure 1D). This insert hybridized with the *dTph1* (*defective transposon Petunia hybrida1*) transposable element (data not shown). In both full red and stable pale revertant alleles of *an9-T3529*, the *dTph1* element had excised (see below). Sequence analysis showed that the *dTph1* element in *an9-T3529* was 284 bp and had inserted at position 529. The insert is highly homologous to the *dTph1* elements found in other mutable loci, such as *dfrC* (*dihydroflavanol reductase C*; Gerats et al., 1990), *rt* (*rhamnosyl transferase*; Kroon et al., 1994), *nam* (*no apical meristem*; Souer et al., 1996), and *an3* (Van Houwelingen et al., 1998). The insertion in *an9-W2463* was 800 bp long and displayed high homology to the termini of *dTph1* (Figure 1E). Upon screening the available sequence databases, we found no significant homology with other sequences, except for a highly similar petunia transposon that had been designated *dTph4* (Renkens et al., 1996). Analysis of these two mutable alleles confirmed that the *difl* cDNA corresponds to the transcript from the *An9* locus.

The *An9* Gene Shares Homology with GSTs

Because the original *difl* cDNA was not full length, we purified nine cross-hybridizing cDNA clones from a petunia cDNA library prepared from the wild-type *An9* line V26. All nine clones survived a high-stringency wash. Three clones were subcloned into plasmid vectors and used for sequence analysis. The two shorter clones measured 883 bp; the longer clone was 962 bp in length. An identical, large open reading frame was present in the long and short cDNAs; in the longer clone, polyadenylation occurred at a different position (Figure 2A). This open reading frame predicts a 232–amino acid protein. Sequence analysis of PCR amplified genomic fragments showed that the *An9* reading frame was interrupted by two introns (Figure 2A). Searches of the SWISS-PROT database showed that the protein encoded by *An9* has high similarity with GSTs, particularly with the plant type I genes. Furthermore, the positions of the two *An9* introns match those of all other type I GST genes (Droog et al., 1995).

To visualize the evolutionary distances among known plant GSTs, we constructed a phylogenetic tree by using several of the type I and type III genes (Figure 2B). Figure 2C shows an alignment of a number of type I and type III plant GSTs. Only a few residues are shared by both type I and type III GSTs, and similarity is highest in the first half of the

Figure 2. (continued).

(B) Phylogenetic tree of several plant type I and type III GSTs. The length of the horizontal lines connecting the sequences is proportional to the estimated genetic distance between these sequences.

(C) The top grouping provides a comparison of petunia AN9 to the type I GSTs, H36860 from Arabidopsis (At-H36860), ParB from tobacco (Nt-ParB; Takahashi and Nagata, 1992), GSTI and GSTIII from maize (Zm-GSTI and Zm-GSTIII; Shah et al., 1986; Grove et al., 1988), GSTIV from maize (Zm-GSTIV; Jepson et al., 1994), GSTA1 from wheat (Ta-GSTA1; Dudler et al., 1991), and a GST from *Silene cucubalus* (Sc-GST; Kutchan and Hochberger, 1992). The group below shows a comparison of maize BZ2 to the type III GSTs GST26A from soybean (Gm-HSP26A, the name in GenBank; Czarnecka et al., 1988), PRP1 from potato (St-PRP1; Taylor et al., 1990), and GST1-1, GST2-1, and ParA from tobacco (Nt-ParA, Nt-GST1-1, and NT-GST2-1; Takahashi et al., 1989; Droog et al., 1993). Amino acids that are conserved in $>$50% of the sequences within each group are black boxed. Dashes indicate gaps in the sequence to allow for maximal alignment. There are six residues that are conserved completely in this comparison and occur at the following positions of the AN9 protein: R(16), P(54), S(71), W(103), D(137), and W(166).

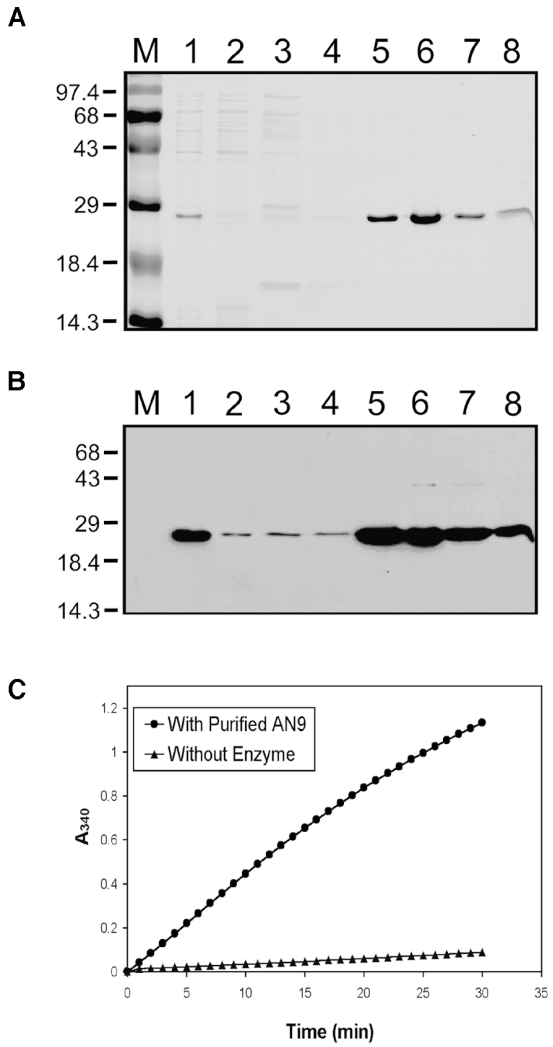

Figure 3. Purification of *An9* and Assay of Enzyme Activity in Vitro.

(A) Coomassie Brilliant Blue R 250–stained SDS–polyacrylamide gel of 6xHis *An9* expressed in *E. coli.* Lane 1 contains total soluble protein; lane 2, flowthrough; lanes 3 and 4, column wash; lanes 5 through 8, elution buffer; and lane M, molecular mass markers given in kilodaltons at left. Gel loading was normalized to protein concentration with the exception of lane 4, which was loaded with the same volume as lane 3.

(B) Immunodetection of 6xHis-tagged construct with anti-6xHis antibody. The SDS–polyacrylamide gel was loaded as described in **(A)**.

(C) Measurement of the GST activity of purified 6xHis–AN9. The increase in absorbance at 340 nm results from the conversion of CDNB to DNP-GS. The triangles are data from a control reaction without enzyme. The circles are data from a reaction containing 9.2 μg of purified protein. Data represent the mean of three replicates.

protein. The petunia *An9* gene is most closely related to the Arabidopsis EST *H36860* and falls within the class of type I GSTs. In contrast, *Bz2* is a type III GST. *An9* is related more to the type I maize genes *GSTI*, *GSTIII*, and *GSTIV* (~33% identity over the whole protein) than it is to *Bz2* (22% amino acid identity in the first half of the protein but only 12% overall). Based on the low sequence identity of type I and type III GSTs, we hypothesized that they diverged from a common ancestral gene before *An9* and *Bz2* evolved into the specialized angiosperm GSTs that are now required for the efficient transport of anthocyanins to the vacuole.

An9 Shows GST Activity in Vitro

The *An9* cDNA was cloned into vector pQE30, yielding a construct under the control of the *lac* promoter, in which six histidine residues were added to the N terminus of AN9. This construct was expressed in *Escherichia coli.* Induced cells produced a soluble protein of ~26 kD, the predicted size of the *An9* gene product (Figure 3A, lane 1). Purification over a Ni-NTA column yielded a single protein of the same size (Figure 3A, lanes 5 to 8). Protein gel blotting demonstrated that the purified protein contained the 6xHis tag (Figure 3B). Crude extract from induced cells lacking the His–*An9* construct did not cross-react with the anti-6xHis tag antibody (data not shown).

To determine whether the purified AN9 protein was a GST, we tested its ability to conjugate GSH to the "universal" substrate, 1-chloro-2,4-dinitrobenzene (CDNB). Most but not all GSTs catalyze the conversion of CDNB to dinitrophenol–glutathione (DNP-GS), with a subsequent increase in absorbance at 340 nm (Holt et al., 1995; Marrs, 1996). A comparison of crude extracts from induced *E. coli* cells demonstrated that cells containing the 6xHis–*An9* construct had significant GST activity, whereas cells without this construct showed no significant increase over the nonenzymatic formation of DNP-GS (data not shown). In the presence of purified AN9, the rate of conversion of CDNB to DNP-GS was >10-fold higher than was the spontaneous formation of DNP-GS (Figure 3C). The estimated V_{max} of AN9 was found to be 3.5 μmol of CDNB conjugated per minute per milligram of protein, which is similar in magnitude to the activity found for other type I GSTs (Zettl et al., 1994; Holt et al., 1995).

An9 and *Bz2* Complement Reciprocally

To examine the in vivo function of *An9*, we tested the ability of the *An9* cDNA to functionally complement the maize *bz2* mutation. Microparticle bombardment was chosen as the functional assay over stable transformation for its rapidity, ease, and reproducibility. The cauliflower mosaic virus (CaMV) 35S::An9::Nos construct, incorporating the *An9* cDNA, was precipitated onto gold particles and bombarded into *bz2* maize kernel aleurones. After 48 hr, the aleurones

were examined. In the positive control, the introduction of *Bz2* on plasmid 35S::Adhi::Bz2::Nos yielded "spots" of one to 20 cells that are strongly pigmented and easily visible against the background of tan, *bz2* aleurone cells (Figure 4C and Table 1). The bombarded *An9* construct yielded anthocyanin expression slightly below 35S::Adhi::Bz2::Nos controls (Figure 4E). Individual spot intensity was the same as that of *Bz2*; however, the spot sizes were generally smaller. As a negative control, the plasmid without an insert was bombarded into *bz2* aleurones, and no purple spots were ever seen (Figure 4A and Table 1). Interestingly, the pigmented spots indicative of functional complementation have a dark purple center and a halo of paler but distinctly pigmented cells. The halos often extend for three or four cells encircling the dark center. Such halos have been observed

Table 1. Quantification of Colored Sectors after Particle Bombardment with GST Constructs

	Sectors/Field [a]			
GST Construct	Small (1 or 2 cells)	Medium (3 to 7 cells)	Large (>7 cells)	Total[b]
Control plasmid	0	0	0	0
35S::Adhi::Bz2::Nos	3.23	2.42	9.31	14.96 ± 2.83
35S::GstI::Nos	0	0	0	0
35S::GstIII::Nos	5.11	0.29	0	5.4 ± 0.77
Ubi::Ubii::GstIV::Nos	0	0	0	0
35S::An9::Nos	1.75	1.33	0.38	3.46 ± 1.56
35S::GmGST26A::Nos	1.97	0.22	0	2.19 ± 0.73
35S::EST H36860::Nos	0	0	0	0

[a] Sectors represent the average number of red cells per 1-mm^2 field scored by three observers, as described in Methods.
[b] Total sectors are given with standard deviation of measurements by three observers.

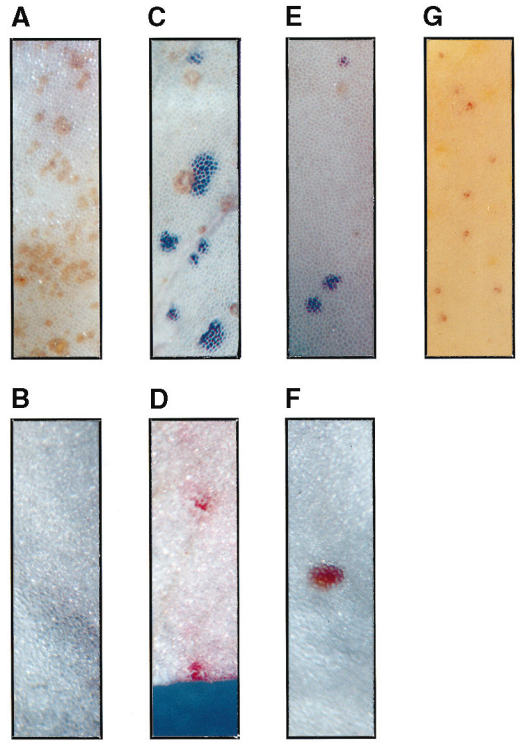

Figure 4. Complementation by Microprojectile Bombardment.

(A) Control pBluescript KS+ bombarded into *bz2* aleurones. Tissue damage resulting from particle impact produced the brown areas.
(B) Control plasmid bombarded into *an9* corolla.
(C) 35S::Adhi::Bz2::Nos bombarded into *bz2* aleurone.
(D) 35S::Adhi::Bz2::Nos bombarded into *an9* corolla.
(E) 35S::An9::Nos bombarded into *bz2* aleurone.
(F) 35S::An9::Nos bombarded into *an9* corolla.
(G) 35S::GSTIII::Nos bombarded into *bz2* aleurone.
Magnification is ×20 in **(A)** to **(G)**. Differences in the background color in the aleurone photographs resulted from natural variation in the beige pigmentation in *bz2* kernels.

previously after excision of *Mutator* (*Mu*) or *Dissociation* (*Ds*) elements from mutable *bz2* alleles (Levy and Walbot, 1990).

We also performed the reciprocal experiment: a constitutively expressed *Bz2* cDNA was bombarded into stable, recessive *an9* flower buds. Flower petals were examined under a microscope for anthocyanin vacuolarization 48 hr after bombardment. Red spots of anthocyanin (Figure 4D) similar to those observed after 35S::An9::Nos bombardment (Figure 4F) were detected. When compared with the borders of wild-type revertant sectors on spotted *an9-T3529* or *an9-T2463* flowers, the spots are equally diffuse but less intense (Figures 1A and 1B, top). The negative control, plasmid with no insert, yielded only a bleaching of the locally bombarded area as a result of cellular necrosis (Figure 4B). Taken together, these data show that *An9* and *Bz2*, despite their high amino acid divergence, perform similar functions in vivo, namely, the conjugation of glutathione to anthocyanins to facilitate the transport of this pigment into the vacuole.

Despite the ability of *An9* to complement *bz2* aleurone tissue, indicating in vivo recognition of C3G, the AN9 protein that was produced in electroporated maize cells, in recombinant *E. coli*, or in vitro failed to efficiently and reproducibly conjugate GSH to C3G in an in vitro assay. Coleman et al. (1997) noted a similar phenomenon with other GSTs. Monochlorobimane (BmCl) fluoresces when conjugated to GSH at the allylic chloride of BmCl. Abundant in vivo fluorescence in plant cell vacuoles has been determined to be the result of GSH conjugation in the cytoplasm and the transport of the glutathionated conjugate into the vacuole. However, purified total plant GSTs and total cellular lysates are incapable of conjugating BmCl to glutathione in vitro. Coleman et al. theorized that product inhibition is responsible for the lack of in vitro conjugation and that the rapid

sequestration of the glutathionated conjugate in the vacuole in vivo relieves this inhibition.

Substrate Specificity of Different GSTs

On the basis of amino acid homology and overall structure, the soybean type III GST *GmGST26A* is related to *Bz2* (Figures 2B and 2C). Even though *GmGST26A* has not been associated with the anthocyanin pathway, we were curious whether it could also complement the *bz2* mutation. A 35S::GmGST26A::Nos construct was bombarded into *bz2* aleurones on three separate occasions. Within 48 hr, complementation was observed. The frequency of purple spots was less than that observed with *An9*, and the size of the spots was always smaller (Table 1). Only once did the *GmGST26A* construct result in spots larger than a single dark cell with a surrounding halo of pale purple cells. One interpretation is that the GmGST26A protein can glutathionate anthocyanins, but less efficiently than either BZ2 or AN9, producing less product as assessed by the number of cells being complemented.

Can genes similar to *An9* also complement the *bz2* and *an9* mutations? A search of the GenBank database identified an Arabidopsis EST, *H36860*, as a strong candidate. Of all the genes assayed in this report, *An9* and *H36860* are the most closely related, with 64% predicted amino acid similarity in the first half of the protein and 50% identity overall (Figures 2B and 2C). Sequencing of the EST demonstrated that it is full length and has a polyadenylated tail. To confirm that the gene encodes a functional GST, it was cloned into a CaMV 35S expression cassette and assayed after electroporation into both maize Black Mexican Sweet protoplasts and tobacco BY2 protoplasts. Cell extracts with the expressed EST–H36860 protein can conjugate GSH to CDNB at a level above background, confirming that the EST cDNA is a functional GST (data not shown). A CaMV 35S::H36860::Nos construct was then bombarded into *bz2* aleurones on three separate occasions. No anthocyanin complementation was ever observed (Table 1).

We previously reported that kernels on a *bz2* ear are occasionally pink, indicating a low level of anthocyanin sequestration in the vacuole even in the absence of the GST encoded by *Bz2* (Walbot et al., 1994). Given that substrate specificity for GSTs can be broad, we hypothesized that at least one of the known maize type I GST genes could encode an enzyme capable of glutathionating C3G, albeit at a much lower efficiency. To test this hypothesis, we made constructs utilizing all three cloned maize type I GSTs, using cDNAs for *GSTI*, *GSTIII*, and *GSTIV*, and bombarded them into *bz2* aleurones three times. As before, we scored anthocyanin vacuolarization after 48 hr. Only CaMV 35S::GSTIII::Nos complemented the *bz2* mutation. The frequency of complementation was slightly higher than that observed for the *An9* construct, but the sectors were much smaller (Table 1) and the intensity of pigment was significantly lower (Figure 4G).

Sequence Analysis of *An9* and *Bz2* Revertants

By using x-ray crystallography, GSTs of diverse origin have been shown to all share a common structure in the N-terminal half of the protein (Pemble and Taylor, 1992; Lee et al., 1995; Reinemer et al., 1996). The catalytic glutathione site involves the first 77 residues in the amino half of the protein, designated as domain I. It is not known how the diverse substrates of GSTs are recognized. To determine which part of the AN9 and BZ2 proteins are required for their GST activity with anthocyanins in vivo, we analyzed various insertion and excision alleles of *Bz2* and *An9* (Figures 5A and 5B, respectively). Sequence analysis of one wild-type revertant *An9* allele indicated that perfect excision of the *dTph1* element had occurred, restoring the original reading frame. Two independent, stable, and recessive *an9* alleles were also analyzed. One, *V503-9*, contains a novel stop codon created within the *dTph1* excision footprint at position 170; the lack of function of this product indicates that the C-terminal third of the protein is required for proper function. In the second stable and nonfunctional allele, *V503-MA*, two amino acids were added to the protein, leaving the open reading frame intact. These two amino acids alter the spacing between an aspartate residue at position 166 that is conserved in all

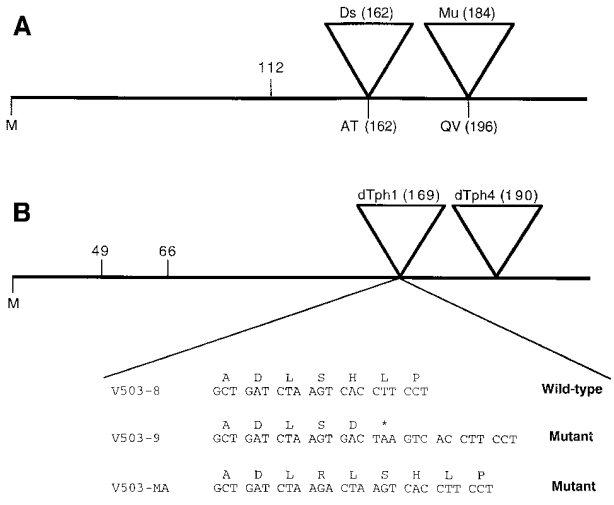

Figure 5. Molecular Analysis of *bz2* and *an9* Alleles.

(A) The junction between exons I and II of *Bz2* is marked at codon position 112. Transposable element insertions are noted above the gene, and amino acid insertions are noted below the gene. Codon positions are in parentheses.

(B) The positions of intron I (at codon position 49) and intron II (at codon position 66) in *An9* are marked. Transposable element insertions are shown at positions 169 and 190. The sequences of one revertant allele (*An9-V503-8*) and two stable recessive alleles (*an9-V503-9* and *an9-V503-MA*) derived after *dTph1* excision are shown below the diagram.

GSTs and a histidine residue at position 169 that is conserved in most type I GSTs.

Revertants of *Mu1* and *Ds* insertions in *Bz2* mutable alleles also were analyzed (Figure 5). *Mu1* is inserted at codon position 184; one fully functional revertant has an extra proline residue. The *Ds1* insertion is at position 162. Four fully functional revertants each have a two–amino acid insertion as compared with the wild type; this insertion is created as part of the 8-bp host sequence duplication. Interestingly, the parental alleles of *bz2*::*Mu1* (*Bz2* Robertson) and *bz2*::*Ds* (*Bz2* Neuffer) are distinguished by two distinct two–amino acid deletions: the Robertson allele is missing an A and T duplication at position 162, and the Neuffer allele is missing Q and V at position 196. The net effect is to maintain the spacing before 162 (T) and after 196 (Q) in these two wild-type alleles. The new *Ds1*-derived revertant alleles alter this spacing by two amino acids, indicating some flexibility within this region.

DISCUSSION

The anthocyanin pathway responsible for the red-blue pigments in many flowering plants has been studied intensively both genetically and molecularly (reviewed in Dooner et al., 1991; Koes et al., 1994; Holton and Cornish, 1995). A consequence of anthocyanin synthesis is that toxic anthocyanins could accumulate in the cytoplasm of the cell. Anthocyanins are stabilized and detoxified by transport to the vacuole. This most likely is accomplished by a mechanism present in all organisms: glutathionation of bioreactive molecules and active transport of the conjugates through a membrane by a GS-X pump requiring Mg-ATP. Recently, we showed that the *Bz2* gene of maize encodes a type III GST that is required for the conjugation of glutathione to anthocyanins (Marrs et al., 1995). In the present study, we demonstrate that the petunia gene *An9* encodes a type I GST and appears to perform a function similar to BZ2. Our finding that distinct GSTs can result in anthocyanin vacuolarization is discussed further with respect to evolution and substrate specificity.

An9 Is a Type I GST Gene

We cloned the petunia *An9* gene and identified it as a type I GST based on its DNA sequence. We have shown that purified AN9 catalyzes the glutathionation of CDNB in vitro and that it is active with anthocyanin substrate in vivo. *An9* had previously been shown to encode a protein required at a late step in the petunia anthocyanin pathway (Gerats et al., 1982), as occurs with *Bz2* in maize. Stable recessive alleles of both genes do not alter anthocyanin structure, but total accumulation is decreased. Visible anthocyanin in *bz2* mutant cells is in the cytoplasm rather than in the vacuole (Marrs et al., 1995). The pink-tinged *an9* petunia petals are so faintly colored that it is not possible to detect localization of the pigment.

The sequence of *An9* is highly homologous to several type I GST genes but shows less homology with the type III GST gene *Bz2.* Alignments of *Bz2* and *An9* to other plant GST sequences show conserved domains, particularly in the N-terminal half of domain I. This suggests that a universal trait of GSTs—their recognition of GSH and/or their ability to dimerize—is contained within this region. Crystallographic analysis of animal GSTs (Pemble and Taylor, 1992; Lee et al., 1995) and an Arabidopsis GST corroborates this idea (Reinemer et al., 1996). Functional GST dimers contain four GSH binding sites; however, only one binding site of each monomer is involved directly in the conjugation of GSH to the substrate. In the Arabidopsis GST (Reinemer et al., 1996), residues 1 to 77 cooperate to form the catalytic GSH binding site. A large cavity in the C-terminal half of the protein, domain II, is proposed to be the substrate recognition domain. By comparing the amino acid sequences of those GSTs that complement the *bz2* mutation, we found no similar amino acid motifs that might constitute a conserved anthocyanin binding site in domain II. The second exon of *Bz2* showed limited homology to cytochrome P450 monooxygenases and steroid binding protein genes (data not shown). These similarities may be an indicator of the capacity to recognize heterocyclic compounds, such as anthocyanin. At the very least, the C-terminal half of both AN9 and BZ2 is critical for either function or protein stability because transposon insertional mutations in this half of either gene eliminate activity.

Although *An9* and *Bz2* belong to distinct classes of GSTs, they perform similar functions. The *An9* cDNA complemented the maize *bz2* mutations and vice versa (Figure 4 and Table 1). This indicates that the diverse anthocyanin structures found in maize and petunia are recognized by both enzymes. Of course, we do not know the full range of in vivo substrates for either BZ2 or AN9. Considering the precedent for relaxed substrate specificity in GSTs, it is reasonable to anticipate that many of the thousands of flavonoids found in plants are recognized by these enzymes.

Diverse GSTs Complement the *bz2* Mutation

Complementation tests also were performed with several GSTs not associated with the anthocyanin pathway. Soybean *GmGST26A* and maize *GSTIII*, which are type III and type I GSTs, respectively, both restored vacuolar pigment localization in *bz2* aleurones. The characteristics of the revertant spots suggest that these GSTs can recognize anthocyanins, but with lower efficiency. Endogenous recognition of C3G by GSTIII, a constitutive enzyme, must be very low to allow a bronze phenotype in a *bz2* mutant background. *GmGST26A* is the closest identified relative of *Bz2. GmGST26A* and *Bz2* are the same size, they share an intron in the identical location, and their gene expression is modulated by similar environmental stimuli, including the unusual feature of splicing

failure during exposure to heavy metals (Czarnecka et al., 1988; Hagen et al., 1988; Nash and Walbot, 1992; Marrs and Walbot, 1997). At the amino acid sequence level, the proteins are 38% identical in exon 1 but very distinct in exon 2 (domain II), where substrate recognition is presumed to occur.

Other type I GST genes, including maize *GSTI*, *GSTIV*, and the Arabidopsis EST *H36860*, failed to confer anthocyanin sequestration in *bz2* aleurones. These three noncomplementing GSTs are related more closely to *An9* than is maize *GSTIII.* At this time, we have no rule for predicting which plant GSTs can recognize anthocyanins as substrates. Individual GSTs metabolize diverse substrates, but our evidence demonstrates that individual enzymes also are somewhat restricted in substrate specificity. Even a huge quantity of specific plant GSTs (present naturally or by transformation) is insufficient to complement the *bz2* mutation, reinforcing the conclusion that GST substrate specificity is broad but not indiscriminate.

Evolutionary Implications

From phylogenetic analysis (Figure 2B; Droog et al., 1995; Marrs, 1996), it is clear that plant GSTs are an ancient and highly diverged gene family. The broad substrate specificity of GSTs probably is not the result of indiscriminate enzymes but more likely the result of economics: several enzymes, each scavenging many endogenous substrates, and diverse, exogenous xenobiotics. As each species confronts new endogenous or exogenous cytotoxins, individuals that survive must express a GST with at least minimal detoxification capacity. GSTs probably are recruited from preexisting pathways, and their regulation and enzymatic properties are then selected to create novel substrate specificities or specific tissue distribution.

Angiosperm evolution is paralleled by the evolution of anthocyanins; it is conceivable that preexisting GSTs with some capacity to recognize anthocyanins were utilized to metabolize this new class of chemicals. The recruited GST must have had other cellular roles before the existence of anthocyanins. In the case of maize, a type III GST was recruited for the sequestration (detoxification) of anthocyanins. In petunia, the recruited GST was a type I enzyme. During subsequent evolution, GST promoters and coding regions may have been “fine tuned” to mold function to substrate diversity and expression pattern. In this respect, it is interesting that *Bz2* in maize and *An9* in petunia are now under the control of homologous regulatory genes. In maize, *Bz2* transcription requires the *myb*-type transcription factors *C1* or *Pl* plus a *myc*-type gene, such as *R* or *B* (reviewed in Dooner et al., 1991). In petunia, transcription of *An9* depends on *An1*, *An2*, and *An11* (Quattrocchio et al., 1993). *An2* encodes a *myb*-type transcription factor (Quattrocchio, 1994) that is functionally homologous to *C1* and *Pl* (Quattrocchio et al., 1998).

Is Glutathionation the Last Step in Anthocyanin Pigmentation?

The regulatory genes of the anthocyanin pathway (*R*/*B* and *C1*/*Pl* in maize; *An1*, *An2*, and *An11* in petunia) control pigmentation in a cell-autonomous manner. In unstable mutants of these loci, red revertant cells are found next to completely uncolored mutant cells (Van Houwelingen et al., 1998). Similarly, introduction of these regulatory genes into mutant backgrounds by particle bombardment produces discrete revertant cells on a pigment-free background (Ludwig et al., 1990; Quattrocchio et al., 1998).

In contrast, mutable alleles of *An9* and *Bz2* yield red-purple revertant cells that are surrounded by a halo of lighter colored cells. Also, colored cells in which the *bz2* or *an9* mutation is complemented via particle bombardment are surrounded by a pale pigmented halo. In both maize and petunia, the presence of a zone of diffusion around revertant cells is a feature of all mutable alleles of structural genes in the anthocyanin pathway (McClintock, 1951; Rhoades, 1952; Levy and Walbot, 1990; Van Houwelingen et al., 1998). This observation applies for genes encoding soluble proteins such as sugar transferases (*Bz1* in maize; *Rt* in petunia) and for the membrane-localized *Ht1* gene product (Stotz et al., 1985), a cytochrome P450 monooxygenase (F. Brugliera, personal communication). Such evidence indicates that the structural genes of the anthocyanin pathway control pigmentation in a non-cell-autonomous manner. We conclude, therefore, that either the enzymes encoded by the structural genes or the anthocyanin precursors can diffuse from a revertant cell to the surrounding mutant cells.

The ability of anthocyanin intermediates to diffuse from cell to cell has been exploited to determine the order of gene action in maize anthocyanin biosynthesis (Reddy and Coe, 1962). In these experiments, aleurone tissues from kernels carrying different anthocyanin pathway mutations were peeled and pressed together. In all cases, pigmentation was restored in only the aleurone tissue that carried the epistatic mutation, suggesting that intermediates in the pathway and not the enzymes themselves were able to diffuse into adjacent aleurone cells. Because the contacts between cells in a single maize aleurone or petunia petal are at least as intimate as those seen in this experiment, the diffusion of anthocyanin precursors must be one of the factors contributing to the halos seen in unstable mutants and bombardment experiments. Furthermore, chemical feeding studies with maize (McCormick, 1978) and petunia (Kho et al., 1975, 1978) established that anthocyanin precursors can move from cell to cell and that the movement of these molecules is sufficient to explain the zones of diffusion.

If glutathionation of anthocyanins were the last step controlled in the anthocyanin pathway or if the BZ2 and AN9 proteins can move between cells, one would expect to find halos for revertant spots of the regulatory genes that control structural gene expression. In such a scenario, diffusion of either the GSTs or the anthocyanin–GS conjugates from

wild-type cells into neighboring regulatory mutant cells would produce a halo similar to that seen in *gst* mutants. The absence of such halos in unstable regulatory mutants therefore has two implications: (1) diffusion of the relatively large (60-kD) GST dimers does not contribute significantly to halo formation, and (2) glutathionation is not the last step in the anthocyanin pathway controlled by the regulatory genes.

Therefore, we postulate that an additional step after glutathionation is under the control of the anthocyanin regulatory genes. One possibility is that the GSH–anthocyanin conjugate must be processed further before it can be considered irreversibly sequestered in the vacuole. Alternatively, a tonoplast GS-X pump could be under the control of the anthocyanin pathway regulators. Genes encoding ABC-type transporters that recognize glutathione conjugates (GS-X pumps) have been cloned from human cell lines and rats; gene families were defined, with each member expressed in specific tissues (Müller et al., 1994; Paulusma et al., 1996). More recently, two genes encoding ABC-type pumps were cloned from Arabidopsis. Vacuoles isolated from yeast lines expressing one of these GS-X plant pumps effectively sequestered glutathione-conjugated C3G in an in vitro assay (Lu et al., 1997). Biochemical evidence suggests that these ABC-type transporters can be induced in response to treatment with herbicide safeners (Gaillard et al., 1994). It is therefore possible that an anthocyanin- or flavonoid-specific Mg-ATP tonoplast pump exists in plants or that regulators of the anthocyanin pathway increase the expression of a constitutively expressed, more general pump.

METHODS

Isolation of Unstable *an9* Alleles and the Molecular Cloning of *An9*

The *an9-T3529* mutant was isolated by random transposon tagging using the petunia line W138. The *an9-W2463* mutant was found among 150,000 plants in the breeding fields of Novartis Seeds (Enkhuizen, The Netherlands). The initial *difI* cDNA was isolated by differential screening for *Anthocyanin1* (*An1*)-regulated genes, as described by Kroon et al. (1994). To obtain full-length cDNA clones, ~60,000 plaques of a λGEM4 cDNA library from V26 petal RNA were screened with the *difI* cDNA probe. After three rounds of purification, the inserts of three clones were subcloned in EcoRI-SalI–digested pBluescript KS+ (Stratagene, La Jolla, CA). The ends of all three clones were sequenced. RNA isolation and RNA gel blot analysis were performed as described by Van Tunen et al. (1988). Polymerase chain reaction (PCR) primers used for analysis of *An9* alleles were an13-1, 5′-dGGAATTCCCTAACAGGAACAACATT-3′ and an13-3, 5′-dCGGGATCCTTTGTCCCGTACTCC-3′.

Purification of AN9 and Assay of GST Activity in Vitro

The *An9* cDNA was PCR amplified using the primers 5′-CGGGATCCGTTGTTAAAGTTCATGGT-3′ and 5′-CTTCTTCGTTTTCAAACTTCGACCTTAAGG-3′. The fragment was digested with HindIII and BamHI and cloned into pQE30 (Qiagen, Chatsworth, CA); this vector adds a six-histidine (6xHis) N-terminal extension. The resulting construct, AQ1, was electroporated into the M15 pRep4 *Escherichia coli* cell line (Qiagen) and sequenced to ensure that no mutations were introduced during PCR amplification. Cells grown to an OD_{600} of ~0.5 were induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.1 mM and grown for 3 hr at 30°C. Cells were recovered by centrifugation for 10 min at 3000*g*, and the pellet was frozen at −20°C. After resuspension in native lysis buffer, cells were lysed in a French press, and the insoluble material was removed by centrifugation at 10,000*g* for 30 min. The protein was purified according to the manufacturer's protocol (Qiagen) and dialyzed overnight against 1 × PBS, pH 7.4. Protein levels were determined using the dye binding method (Bio-Rad). Loading of SDS–polyacrylamide gels was normalized for protein concentration unless otherwise indicated.

In vitro assays for glutathione *S*-transferase (GST) activity were performed in triplicate on a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA). Purified enzyme was added to 1 mM glutathione in 1 × PBS, pH 7.4. For the control reaction, an equal volume of 1 × PBS, pH 7.4, was added in place of the enzyme extract. 1-Chloro-2,4-dinitrobenzene (CDNB) was added immediately before measurement to a final concentration of 1 mM in a 200-μL reaction volume. Absorbance at 340 nm was used to measure the rate of conjugation over 30 min. The specific activity of purified AN9 was determined according to the method of Holt et al. (1995). Data are the mean of three replicates.

Sequence Analysis

The longest *An9* clone was sequenced completely by using asymmetric PCR with fluorescent M13 primers, using a DNA sequencer (model 370A; Applied Biosystems, Foster City, CA). Sequence alignments were performed using the GeneWorks 2.0 program from IntelliGenetics, Inc. (Palo Alto, CA). Alignments were optimized by hand. The phylogenetic tree was constructed using the tree option within the GeneWorks 2.0 program that uses the unweighted pair group method with arithmetic mean, as described by Nei (1987). The *An9* cDNA has GenBank accession number Y07721. The *dTph4* element sequenced in this study has GenBank accession number Y07722.

The *Arabidopsis thaliana* expressed sequence tag (EST) (GenBank accession number H36860) was sequenced using dye terminator cycle sequencing with AmpliTaq DNA polymerase, FS (Perkin-Elmer, Foster City, CA) with T7, SP6, and sequence-specific primers. Reactions were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems), and the sequence was analyzed using their Sequence Navigator software. The complete EST sequence was given the GenBank accession number U70672. Primers designed from the Arabidopsis EST *H36860* amplified two yeast artificial chromosomes (YACs): yUP1B6 and yUP4G12. Both yUP1B6 and yUP4G12 are anchored on the top arm of chromosome 3 of the Arabidopsis physical map near the molecular marker *nga172*.

Sequence-specific primers (5′-TCAACCGGTGGTGGAATGAG-3′ and 5′-AATGGAGTACAAGCGTCAAG-3′) were made to the Arabidopsis EST *H36860* and used to amplify DNA prepared from pools of the Arabidopsis YAC UP library (Matallana et al., 1992). Candidate YACs that contained the Arabidopsis EST were grown individually and verified for amplification of the Arabidopsis EST.

Generation of Chimeric GST Constructs for Plant Expression Studies

In all cases, cDNAs of GST genes were used in plasmid constructs. *GSTI* and *GSTIV* were kindly supplied by I. Jepson (Zeneca Agrochemicals, Berkshire, UK). The cauliflower mosaic virus (CaMV) 35S::GSTI::Nos construct was carried in a pBin-based vector with a polyadenylation region from the nopaline synthase gene (Nos). The CaMV 35S::GSTI::Nos cassette was subcloned as an EcoRI-HindIII fragment into the pBluescript KS+ vector. The *GSTIV* expression cassette, Ubi::Ubii::GstIV::Nos, was used in the form in which it was supplied; the pUC19-based vector contains a maize ubiquitin promoter, the maize ubiquitin intron, the cDNA of maize *GSTIV* cDNA, and the Nos polyadenylation region. *GSTIII* was kindly supplied by K. Timmerman and C.-P.D. Tu (Pennsylvania State University, University Park, PA). The *GSTIII* cDNA clone was removed as a 0.92-kb EcoRI fragment. This fragment was blunt ended with the Klenow fragment of DNA polymerase I, BamHI linkers were added, and the resulting fragment was ligated into the BamHI site of pJD288 to create 35S::GSTIII::Nos. pJD288 is a CaMV 35S::Polylinker::Nos vector generated in our laboratory (Luehrsen et al., 1992). Similarly, the Arabidopsis EST H36860 was cloned into this vector to create 35S::EST H36860::Nos. The *An9* cDNA was subcloned as a BamHI-KpnI fragment into a CaMV 35S::Nos vector in pBluescript KS+ to create 35S::An9::Nos. The *Bz2* expression cassette 35S::Adhi::Bz2::Nos has the CaMV 35S promoter, followed by intron 1 of maize *Alcohol dehydrogenase1* (*Adh1*), the *Bz2* cDNA, and the Nos polyadenylation sequence (Nash and Walbot, 1992). The structure of the *GmHSP26A* gene construct is as published in Hagen et al. (1988); the vector contains the 35S promoter and the soybean cDNA (renamed 35S::GmGST26A::Nos for this study).

Because the ubiquitin and CaMV 35S promoters are so strong, it is assumed that the predominant GST dimer produced in the transient assay is the homodimer of the construct. A low level of heterodimerization of the test GST with endogenous GSTs may occur. Although 1% of soluble leaf protein is GSTs, not all are potential dimerization partners, and presumably the majority of endogenous GSTs are already dimerized. *An9*, *GmGST26A*, *GSTI*, and *GSTIII* are all under the identical form of the very strong promoter CaMV 35S. Consistent differences in complementation level were always noted for each construct—from strong with *An9* to none with *GSTI.* This indicates that these enzymes have different catalytic capacities on anthocyanin. The most dramatic example that complementation-by-overexpression has definite limits is found with the *GSTIV* construct. This expression cassette uses the maize ubiquitin promoter and intron, which has been well documented to yield much stronger expression in monocots than does the CaMV 35S promoter (Christensen and Quail, 1996), yet no complementation was ever noted.

Bombardment

Nine kernels of the Maize Genetics Cooperation Stock Center (Urbana, IL) *bz2* deletion stock homozygous for the *anther ear* (*an*) to *bz2* deletion were used for each test construct. Twenty-four hours before bombardment, the seeds were covered with a moist paper towel and allowed to hydrate in a covered Petri dish. Immediately before bombardment, the pericarps were removed to expose the light buff to brown-gray aleurone tissue. Twenty-five micrograms of test plasmid was precipitated onto 1-μm spherical gold beads (Bio-Rad). The bombardments were performed according to Wan and Lemaux (1994). Three kernels were bombarded at a time, with three replicates for each test construct. The kernels were then returned to the Petri dish and placed under the moist towel. The kernels were incubated on a benchtop for 48 hr and then examined at ×30 magnification for anthocyanin accumulation in the vacuole. To quantify the level of complementation, the number of revertant sectors found in 1-mm^2 fields (~4000 cells) was determined. Fields were selected to cover the sites of greatest DNA delivery, as assessed by tissue damage resulting from the impact of the gold particles. Revertant sectors were divided into three size classes based on the number of contiguous cells showing vacuolarized pigment. The results represent an average of the number and size of revertant sectors recorded by three independent observers.

The stable recessive *an9* allele, *V503-MA*, was used for the bombardments. Flower buds to be bombarded were harvested 2 days before any anthocyanin pigment was visible. The green buds were cut and immediately stabbed into Petri plates containing solid media. The media was 1% GelRite agarose (Kelco, San Diego, CA), pH 5.5, in water. The buds were left on the plates for 24 hr. Then, the upper third of the inside of the corolla was excised from the bud for bombardment. Bombardment was only successful in tissue from buds that had "cured" in this manner for 1 day. After bombardment, the flower petals were returned to the agarose and incubated on a benchtop for 48 hr. The petals were screened for anthocyanin accumulation at ×30 magnification.

Protoplast Electroporations and GST Assays

The maize Black Mexican Sweet cell suspension culture was electroporated with 0, 10, or 25 μg of test plasmid, as described by Carle-Urioste et al. (1995). The tobacco BY2 cell suspension culture was electroporated similarly but with two modifications. The tobacco POR medium contained 400 mM mannitol as an osmoticum; during electroporation, polyethylene glycol 3350 poration was added to the poration medium (Sigma) to a final concentration of 10%. After electroporation, both types of cells were gently shaken at 20 rpm in the dark at room temperature for 48 hr before being assayed for GST activity. The GST assays were performed as described by Marrs et al. (1995).

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REFERENCES

Bartling, D., Radzio, R., Steiner, U., and Weiler, E.W. (1993). A glutathione *S*-transferase with glutathione peroxidase activity from *Arabidopsis thaliana*: Molecular cloning and functional characterization. Eur. J. Biochem. **216,** 579–586.

Carle-Urioste, J.C., Marrs, K., Bodeau, J., and Walbot, V. (1995). Gene transfer to protoplasts: Transient gene expression analysis. In Gene Transfer to Plants, I. Potrykus and G. Spangenberg, eds (Berlin: Springer-Verlag), pp. 106–111.

Christensen, A.H., and Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. **5,** 213–218.

Coleman, J.O.D., Randall, R., and Blake-Kalff, M.M.A. (1997). Detoxification of xenobiotics in plant cells by glutathione conjugation and vacuolar compartmentalization: A fluorescent assay using monochlorobimane. Plant Cell Environ. **20,** 449–460.

Czarnecka, E., Nagao, R.T., and Key, J.L. (1988). Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat-shock protein of soybean: Heavy-metal–induced inhibition of intron processing. Mol. Cell. Biol. **8,** 1113–1122.

Dooner, H.K., Robbins, T.P., and Jorgensen, R.A. (1991). Genetic and developmental control of anthocyanin biosynthesis. Annu. Rev. Genet. **25,** 173–200.

Droog, F.N.J., Hooykaas, P.J.J., Libbenga, K.R., and Zaal, E.J. (1993). Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferases and one member indeed shows *in vitro* GST activity. Plant Mol. Biol. **21,** 965–972.

Droog, F.N.J., Hooykaas, P.J.J., and Van der Zaal, B.J. (1995). 2,4-Dichlorophenoxyacetic acid and related chlorinated compounds inhibit two auxin-regulated type III tobacco glutathione *S*-transferases. Plant Physiol. **107,** 1139–1146.

Dudler, R., Hertig, C., Rebman, G., Bull, J., and Mauch, F. (1991). A pathogen-induced wheat gene encodes a protein homologous to glutathione *S*-transferases. Mol. Plant-Microbe Interact. **4,** 14–18.

Edwards, R., Blount, J.W., and Dixon, R. (1991). Glutathione and elicitation of the phytoalexin response in legume cell cultures. Planta **184,** 403–409.

Gaillard, C., Dufaud, A., Tommasini, R., Kreuz, K., Amrhein, N., and Martinoia, E. (1994). A herbicide antidote (safener) induces the activity of both the herbicide detoxifying enzyme and of a vacuolar transporter for the detoxified herbicide. FEBS Lett. **352,** 219–221.

Gerats, A.G.M., de Vlaming, P., Doodeman, M., and Schram, A.W. (1982). Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of *Petunia hybrida.* Planta **155,** 364–368.

Gerats, A.G.M., Hiuts, H., Vrijlandt, E., Maraña, C., Souer, E., and Beld, M. (1990). Molecular characterization of a nonautonomous transposable element (*dTph1*) of petunia. Plant Cell **2,** 1121–1128.

Grove, G., Zarlengo, R.P., Timmerman, K.P., Li, N., Tam, M.F., and Tu, C. (1988). Characterization and heterospecific expression of cDNA clones of genes in the maize GSH S-transferase multigene family. Nucleic Acids Res. **16,** 425–428.

Hagen, G., Uhrhammer, N., and Guilfoyle, T.J. (1988). Regulation of expression of an auxin-induced soybean sequence by cadmium. J. Biol. Chem. **263,** 6442–6446.

Holt, D.C., Lay, V.J., Dinsmore, A., Jepson, I., Bright, S.W.J., and Greenland, A.J. (1995). Characterization of safener-induced glutathione S-transferase isoform II from maize. Planta **196,** 295–302.

Holton, T.A., and Cornish, E.C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7,** 1071–1083.

Itzhaki, H., and Woodson, W.R. (1993). Characterization of an ethylene-responsive glutathione S-transferase gene cluster in carnation. Plant Mol. Biol. **22,** 43–58.

Jepson, I., Lay, V.J., Holt, D.C., Bright, S.W.J., and Greenland, A.J. (1994). Cloning and characterization of maize herbicide safener-induced cDNAs encoding subunits of glutathione *S*-transferase isoforms I, II, and IV. Plant Mol. Biol. **26,** 1855–1866.

Kho, K.F.F., Bennink, G.J.H., and Wiering, H. (1975). Anthocyanin synthesis in a white flowering mutant of *Petunia hybrida* by a complementation technique. Planta **127,** 271–279.

Kho, K.F.F., Blosman-Louwen, A.C., Vuik, J.C., and Bennink, G.J.H. (1978). Anthocyanin synthesis in a white flowering mutant of *Petunia hybrida*. II. Accumulation of dihydroflavonol intermediates in white flower mutants, uptake of intermediates in isolated corollas and conversion into anthocyanins. Planta **135,** 109–118.

Kim, C.-S., Kwak, J.-M., Nam, H.-G., Kim, K.-C., and Cho, B.-H. (1994). Isolation and characterization of two cDNA clones that are rapidly induced during the wounding response of *Arabidopsis thaliana.* Plant Cell Rep. **13,** 340–343.

Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Characterization of two cDNAs (ERD11 and ERD13) for dehydration-inducible genes that encode putative glutathione *S*-transferases in *Arabidopsis thaliana* L. FEBS Lett. **335,** 189–192.

Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: Function and evolution. Bioessays **16,** 123–132.

Kroon, J., Souer, E., de Graaff, A., Xue, Y., Mol, J., and Koes, R. (1994). Cloning and structural analysis of the anthocyanin pigmentation locus *Rt* of *Petunia hybrida*: Characterization of insertion sequences in two mutant alleles. Plant J. **5,** 69–80.

Kutchan, T.M., and Hochberger, A. (1992). Nucleotide sequence of a cDNA encoding a constitutively expressed glutathione *S*-transferase from cell suspension cultures of *Silene cucubalis.* Plant Physiol. **99,** 789–790.

Lee, H.-C., Toung, Y.-P.S., Tu, Y.-S.L., and Tu, C.-P.D. (1995). A molecular genetic approach for the identification of essential residues in human glutathione *S*-transferase function in *Escherichia coli.* J. Biol. Chem. **270,** 99–109.

Levy, A.A., and Walbot, V. (1990). Regulation of the timing of transposable element excision during maize development. Science **248,** 1534–1537.

Li, Z.-S., Zhen, R.-G., and Rea, P.A. (1995). 1-Chloro-2,4-dinitrobenzene–elicited increase in vacuolar glutathione-*S*-conjugate transport activity. Plant Physiol. **109,** 177–185.

Li, Z.-S., Alfenito, M., Rea, P.A., Walbot, V., and Dixon, R.A. (1997). Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. Phytochemistry **45,** 689–693.

Lu, Y.P., Li, Z.S., and Rea, P.A. (1997). *AtMRP1* gene of *Arabidopsis* encodes a glutathione *S*-conjugate pump: Isolation and functional definition of a plant ATP-binding cassette transporter gene. Proc. Natl. Acad. Sci. USA **94,** 8243–8248.

Ludwig, S.R., Bowen, B., Beach, L., and Wessler, S. (1990). A regulatory gene as a novel visible marker for maize transformation. Science **247,** 449–450.

Luehrsen, K., DeWet, J., and Walbot, V. (1992). Use of luciferase as a reporter gene. Methods Enzymol. **216,** 397–414.

Marrs, K.A. (1996). The functions and regulation of glutathione *S*-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 127–148.

Marrs, K.A., and Walbot, V. (1997). Expression and RNA splicing of the maize glutathione *S*-transferase *Bronze2* is regulated by cadmium and other stresses. Plant Physiol. **113,** 93–102.

Marrs, K.A., Alfenito, M.R., Lloyd, A.M., and Walbot, V. (1995). A glutathione *S*-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2.* Nature **375,** 397–400.

Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., and Amrhein, N. (1993). ATP-dependent glutathione *S*-conjugate export 'pump' in the vacuolar membrane of plants. Nature **364,** 247–249.

Matallana, E., Bell, C.J., Dunn, P.J., Lu, M., and Ecker, J.R. (1992). Genetic and physical linkage of the *Arabidopsis* genome: Methods for anchoring yeast artificial chromosomes. In Methods in *Arabidopsis* Research, C. Koncz, N.-H. Chua, and J. Schell, eds (Singapore: World Scientific Publishing), pp. 144–169.

McClintock, B. (1951). Chromosome organization and genic expression. Cold Spring Harbor Symp. Quant. Biol. **16,** 13–47.

McCormick, S. (1978). Pigment synthesis in maize aleurone from precursors fed to anthocyanin mutants. Biochem. Genet. **16,** 777–785.

Meyer, R.C., Goldsbrough, P.B., and Woodson, W.R. (1991). An ethylene-responsive gene from carnation encodes a protein homologous to glutathione *s*-transferases. Plant Mol. Biol. **17,** 277–281.

Müller, M., Meijer, C., Zaman, G.J.R., Borst, P., Scheper, R.J., Mulder, N.H., deVries, G.E., and Jansen, P.L.M. (1994). Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. Proc. Natl. Acad. Sci. USA **91,** 13033–13037.

Nash, J., and Walbot, V. (1992). *Bronze-2* gene expression and intron splicing patterns in cells and tissues of *Zea mays* L. Plant Physiol. **100,** 464–471.

Nei, M. (1987). Molecular evolutionary genetics. In Molecular Evolutionary Genetics. (New York: Columbia University Press), pp. 293–298.

Paulusma, G.C., Bosma, P.J., Zaman, G.J.R., Bakker, C.T.M., Otter, M., Scheffer, G.L., Scheper, R.J., Borst, P., and Oude Elferink, R.P.J. (1996). Jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science **271,** 1126–1128.

Pemble, S.E., and Taylor, J.B. (1992). An evolutionary perspective on glutathione transferases inferred from class-Theta glutathione transferase cDNA sequences. Biochem. J. **287,** 957–963.

Quattrocchio, F. (1994). Regulatory Genes Controlling Flower Pigmentation in *Petunia hybrida.* PhD Dissertation (Amsterdam: Vrije Universiteit).

Quattrocchio, F., Wing, J.F., Leppen, H.T.C., Mol, J.N.M., and Koes, R.E. (1993). Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell **5,** 1497–1512.

Quattrocchio, F., Wing, J.F., Van der Woude, K., Mol, J.N.M., and Koes, R. (1998). Analysis of bHLH and MYB-domain proteins: Species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant J. **13,** 475–488.

Reddy, G.M., and Coe, E.H. (1962). Inter-tissue complementation: A simple technique for direct analysis of gene action sequence. Science **138,** 149–150.

Reinemer, P., Prade, L., Hof, P., Neuefeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Keolln, I., Bartunik, H.D., and Bieseler, B. (1996). Three-dimensional structure of glutathione *S*-transferase from *Arabidopsis thaliana* at 2.2 Å resolution: Structural characterization of herbicide-conjugating plant glutathione *S*-transferases and a novel active site architecture. J. Mol. Biol. **255,** 289–309.

Renkens, S., De Greve, H., Beltrán-Herrera, J., Toong, L.T., Deboeck, F., De Rycke, R., Van Montagu, M., and Hernalsteens, J.P. (1996). Insertion mutagenesis and study of transposable elements using a new unstable virescent seedling allele for isolation of haploid petunia lines. Plant J. **10,** 533–544.

Rhoades, M.M. (1952). The effect of the bronze locus on anthocyanin formation in maize. Am. Nat. **86,** 105–108.

Sari-Gorla, M., Ferrario, S., Rossini, L., Frova, C., and Villa, M. (1993). Developmental expression of glutathione-S-transferase in maize and its possible connection with herbicide tolerance. Euphytica **67,** 221–230.

Shah, D.M., Hironaka, C.M., Wiegand, R.C., Harding, E.I., Krivi, G.G., and Tiemeier, D.C. (1986). Structural analysis of a maize gene coding for glutathione–S-transferase involved in herbicide detoxification. Plant Mol. Biol. **6,** 203–211.

Souer, E., Van Houwelingen, A., Kloos, D., Mol, J.N.M., and Koes, R.E. (1996). The *no apical meristem* gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell **85,** 159–170.

Stotz, G., de Vlaming, P., Wiering, H., Schram, A.W., and Forkmann, G. (1985). Genetic and biochemical studies on flavonoid 3′-hydroxylation in flowers of *Petunia hybrida.* Theor. Appl. Genet. **70,** 300–305.

Takahashi, Y., and Nagata, T. (1992). *parB*: An auxin-regulated gene encoding glutathione *S*-transferase. Proc. Natl. Acad. Sci. USA **89,** 56–59.

Takahashi, Y., Kuroda, H., Tanaka, T., Machida, Y., Takebe, I., and Nagata, T. (1989). Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci. USA **86,** 9279–9283.

Takahashi, Y., Hasezawa, S., Kusaba, M., and Nagata, T. (1995). Expression of the auxin-regulated *parA* gene in transgenic tobacco and nuclear localization of its gene products. Planta **196,** 111–117.

Taylor, T.L., Fritzemeyer, K.H., Hauser, I., Kombrink, E., Rohwer, F., Schroder, M., Strittmatter, G., and Hahlbrock, K. (1990).

Structural analysis and activation of a gene encoding a pathogenesis-related protein in potato. Mol. Plant-Microbe Interact. **3,** 72–77.

Van Houwelingen, A., Souer, E., Spelt, C., Kloos, D., Mol, J., and Koes, R. (1998). Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida.* Plant J. **13,** 39–50.

Van Tunen, A.J., Koes, R.E., Spelt, C.E., Van der Krol, A.R., Stuitje, A.R., and Mol, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: Coordinate, light-regulated and differential expression of flavonoid genes. EMBO J. **7,** 1257–1263.

Walbot, V. (1996). Sources and consequences of phenotypic and genotypic plasticity in flowering plants. Trends Plant Sci. **1,** 27–32.

Walbot, V., Benito, M.-I., Bodeau, J., and Nash, J. (1994). Abscisic acid induces pink pigmentation in maize aleurone tissue in the absence of *Bronze-2.* Maydica **39,** 19–28.

Wan, Y., and Lemaux, P.G. (1994). Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. **104,** 37–48.

Zettl, R., Schell, J., and Palme, K. (1994). Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido-[7-^{3}H]indole-3-acetic acid: Identification of a glutathione *S*-transferase. Proc. Natl. Acad. Sci. USA **91,** 689–693.

Zhou, J., and Goldsbrough, P.B. (1993). An *Arabidopsis* gene with homology to glutathione *S*-transferases is regulated by ethylene. Plant Mol. Biol. **22,** 517–523.

Functional Complementation of Anthocyanin Sequestration in the Vacuole by Widely Divergent Glutathione *S*-Transferases

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