# AN9, a Petunia Glutathione *S*-Transferase Required for Anthocyanin Sequestration, Is a Flavonoid-Binding Protein[1]

**Lukas A. Mueller*, Christopher D. Goodman, Rebecca A. Silady, and Virginia Walbot**

Department of Biological Sciences, 385 Serra Mall, Stanford University, Stanford, California 94305–5020

AN9 is a glutathione *S*-transferase from petunia (*Petunia hybrida*) required for efficient anthocyanin export from the site of synthesis in the cytoplasm into permanent storage in the vacuole. For many xenobiotics it is well established that a covalent glutathione (GSH) tag mediates recognition of molecules destined for vacuolar sequestration by a tonoplast-localized ATP-binding cassette pump. Here we inquired whether AN9 catalyzes the formation of GSH conjugates with flavonoid substrates. Using high-performance liquid chromatography analysis of reaction mixtures containing enzyme, GSH, and flavonoids, including anthocyanins, we could detect neither conjugates nor a decrease in the free thiol concentration. These results suggest that no conjugate is formed in vitro. However, AN9 was shown to bind flavonoids using three assays: inhibition of the glutathione *S*-transferase activity of AN9 toward the common substrate 1-chloro 2,4-dinitrobenzene, equilibrium dialysis, and tryptophan quenching. We conclude that AN9 is a flavonoid-binding protein, and propose that in vivo it serves as a cytoplasmic flavonoid carrier protein.

Glutathione *S*-transferases (GSTs) comprise a large family of ubiquitous enzymes; collectively, they constitute about 1% of the soluble protein in photosynthetic plant cells (Hayes and Pulford, 1995; Marrs, 1996). GSTs are required for detoxification of diverse exogenous substrates including many drugs administered to animals and herbicides applied to plants (Sandermann, 1992; Coleman et al., 1997). Enzyme diversity is reflected by the numerous family members and the fact that GSTs function as homo- and heterodimers of 20- to 30-kD subunits. Herbicides and safeners, because of their economic importance, have been the major focus of interest as substrates of plant GSTs. In plants the well-studied detoxification reactions ultimately lead to vacuolar sequestration of substrates conjugated to glutathione (GSH). Little is known, however, about the function of GSTs with endogenous compounds. This is striking because plants synthesize numerous toxic secondary metabolites that are potential GST substrates. These compounds must be compartmentalized for the plant to survive (Walbot, 1996).

Anthocyanins (Fig. 1) are a branch of the family of flavonoids (Holton and Cornish, 1995). They are brightly colored pigments that are normally localized in the vacuole. Depending on constituent substitutions and complexes formed with metal ions and copigments, anthocyanins can appear red, blue, or violet under the low pH, vacuolar conditions. *Bronze2* (*Bz2*) is the last genetically defined locus in the anthocyanin pathway in corn. *bz2* loss-of-function alleles impart a bronze color to affected kernels. In *bz2* tissues anthocyanin accumulates in the cytoplasm where it undergoes oxidation and polymerization reactions (Alfenito et al., 1998); the oxidized products appear brown instead of the bright colors typical of vacuolar anthocyanins. Thus in *bz2* mutants, vacuolar sequestration of anthocyanin pigments is impaired.

In petunia (*Petunia hybrida*), the *An9* gene performs the role analogous to that of *Bz2* in maize. Although BZ2 and AN9 have only 12% amino acid identity, they can reciprocally complement *an9* and *bz2* tissues in particle gun bombardment assays (Alfenito et al., 1998). The AN9 and BZ2 proteins are classified as GSTs based on two criteria. First, they share sequence similarity with other plant and non-plant GSTs; with a single intron, *Bz2* is a typical type-III or $\tau$ plant GST (McLaughlin and Walbot, 1987; Marrs et al., 1995; Marrs, 1996), whereas *An9* has two introns characteristic of type-I or $\phi$ plant GST genes (Alfenito et al., 1998). Second, AN9 and BZ2 can catalyze covalent glutathionation of the common substrate 1-chloro 2,4-dinitrobenzene (CDNB), which is recognized by most, but not all, GSTs (Mannervik and Danielson, 1988). BZ2 protein, when expressed in bacteria, had measurable but low CDNB conjugating activity (Marrs et al., 1995), whereas AN9 had substantial CDNB activity (Alfenito et al., 1998). AN9 was therefore preferred over BZ2 in the present study examining flavonoid-GST interaction.

Because *An9* and *Bz2* encode GSTs, we had a unique opportunity to investigate the action of GSTs

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* Corresponding author; e-mail Lukas_Mueller@mail.com; fax 650–725–8221.

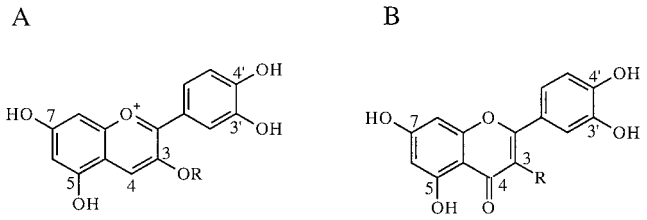


**Figure 1.** Structure of the major flavonoids used in experiments. A, Structure of cyanidin (R = H) and cyanidin 3-glucoside (R = Glc). B, Structure of quercetin (R = OH), luteolin (R = H), and isoquercitrin (R = glucosyl).

on proven endogenous substrates. The precise biochemical role of BZ2 and AN9 in fostering sequestration of anthocyanins to the vacuole has not been thoroughly investigated. It has been assumed, by analogy to the detoxification of herbicides, that these GSTs catalyze conjugate formation between the tripeptide GSH (γ-Glu-Cys-Gly) and anthocyanins such as cyanidin-3-glucoside (C3G). When radiolabeled GSH was incubated with Black Mexican Sweet corn tissue culture cells expressing the anthocyanin pathway, radioactivity colocalized with pigment on two-dimensional thin-layer chromatography (TLC; Marrs et al., 1995), but the putative products have not been further isolated and identified. Moreover there are no reports of naturally occurring conjugates between anthocyanins and GSH or of anthocyanin to Cys, a common breakdown product of xenobiotic conjugates. Hence, this work was initiated to characterize the elusive GSH adducts of C3G using plant enzymes with genetically defined roles, purified after expression in *Escherichia coli*.

Despite their specific name GSTs have many roles. Some isoenzymes have peroxidase (Bartling et al., 1993; Cummins et al., 1999), isomerase (Benson et al., 1977; Hayes and Pulford, 1995), and diverse binding activities (Litwack et al., 1971; Bhargava et al., 1977; Vander Jagt et al., 1985; Hayes and Pulford, 1995). Any of these activities could play a role in the interaction of BZ2 and AN9 with anthocyanins in vivo. Based on our results we propose a new model for the GST required in vacuolar sequestration of flavonoids. We suggest that AN9 and BZ2 are cytoplasmic "escort" proteins for anthocyanin and that sequestration is accomplished without formation of a GSH conjugate in the cytoplasm. In this regard BZ2 and AN9 function as ligandins whose binding activity is required in vivo but which do not directly catalyze any enzymatic conversion (Edwards et al., 2000).

## RESULTS

### Absence of Conjugates in Vitro

In an effort to produce flavonoid-glutathione conjugates in vitro, recombinant AN9 enzyme purified from *E. coli* was incubated with [$^{35}$S]GSH and potential substrates, including the flavonoids quercetin, isoquercitrin, cyanidin, and C3G (Fig. 1). The mixtures were analyzed by HPLC. The results for C3G are shown in Figure 2. After a 2-h incubation, no radioactivity colocalized with the peak absorbing at 510 nm, where red-colored C3G most strongly absorbs (Fig. 2A). No other peak could be detected that absorbed at 510 nm, and the C3G peak was identical in size and elution time whether GSH was added to the reaction or was omitted (Fig. 2B). We conclude that C3G is not modified during the incubation. Two peaks did appear in the radioactivity trace at 3.3 and 4.3 min (corrected for radiodetector offset, see "Materials and Methods"). Fresh, reduced [$^{35}$S]GSH eluted at 3.3 min (corrected for the radiodetector

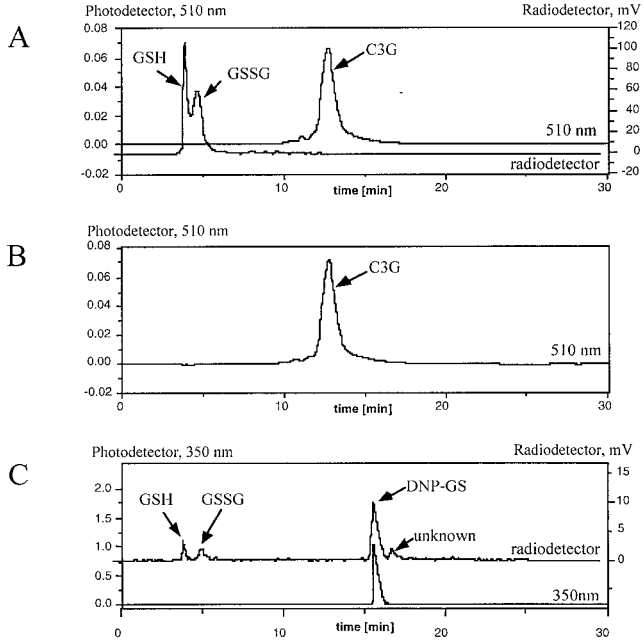


**Figure 2.** HPLC analysis of reaction products with AN9, GSH, and C3G. A, HPLC analysis of reaction products of AN9, GSH, and C3G (see "Materials and Methods" for analysis conditions). Photodetector and radioactivity detector signals are superimposed. On the left axis, units for the absorption at 510 nm are given; on the right, radioactivity detection in millivolts. The 510-nm absorption signal shows C3G, which elutes at about 13 min. The radiodetector signal shows two peaks, which are GSH at 3.3 min, and a peak at 4.3 min, which was also observed after prolonged incubation of GSH alone and when GSH was incubated with $H_2O_2$. We therefore assigned this peak to GSSG. No formation of conjugate is evident from this experiment. Results were analogous when other flavonoids were used. B, HPLC analysis of reaction products when AN9 is incubated with C3G, omitting GSH. The C3G peak is identical in size and elution time as in A, indicating that no C3G substrate disappeared during the incubation shown in A. C, Analysis of the conjugates between GSH and the model substrate CDNB. Unconjugated CDNB absorbs at 280 nm and eluted at 23 min (not shown). The conjugate, DNP-GS, elutes at approximately 16 min and absorbs at 350 nm. Radiolabeled GSH was used for conjugate synthesis. The conjugate was detected both by measuring the absorption at 350 nm and by the radiodetector, as shown. A small peak was detected at approximately 17 min in the radiodetector, which probably corresponds to a GSH conjugate formed with an impurity or breakdown product of CDNB.

offset, data not shown), near the "flow-through" (Fig. 2A). The second peak at 4.3 min appears when GSH is incubated alone for several hours, or when GSH is incubated with $H_2O_2$ (data not shown). We conclude that this peak corresponds to oxidized GSH, the dimeric molecule oxidized glutathione (GSSG; Fig. 2A). In another set of experiments [$^3$H]luteolin and [$^3$H]isoquercitrin were incubated with GSH and AN9, and the products were analyzed with HPLC. Again, no new labeled spots or absorbing peaks could be identified (data not shown). These experiments indicate that no conjugates form between GSH and the flavonoids we tested.

To judge if our detection methods were adequate we tested whether we could analyze the conjugate of CDNB and GSH, dinitrophenyl-glutathione conjugate (DNP-GS), by HPLC. DNP-GS was produced chemically (see "Materials and Methods"). The substrate CDNB is very hydrophobic and elutes near the end of the gradient at 22.7 min (data not shown). Its conjugate, DNP-GS, is rendered less hydrophobic by the GSH moiety; it elutes earlier than CDNB at 15 min, and it absorbs at 350 nm (Fig. 2C, 350 nm trace). When [$^{35}$S]GSH was included in the incubations, radioactivity co-eluted with the DNP-GS peak, as expected for the GSH conjugate of CDNB (Fig. 2C).

Detecting formation of conjugates can be difficult when their properties are unknown. The GSH conjugates of anthocyanins could, for example, be unstable in the acidic conditions used for HPLC analysis. To assess whether conjugates do form, but were undetected by HPLC, we measured thiol concentration during the reaction. The concentration of free thiol group of GSH should decrease upon formation of a conjugate. As expected, the free thiol concentration decreased in enzymatic incubations of AN9 with CDNB and GSH, reaching 25% of the input value within 1 h as DNP-GS is rapidly formed (Fig. 3). In contrast only a very slight decrease of free thiols could be detected when C3G, cyanidin, quercetin, or isoquercitrin were used as substrates. The magnitude of thiol reduction was the same whether enzyme was added or omitted and may therefore represent the spontaneous formation of GSSG. This experiment was repeated under several pH conditions (pH 6.0, 6.5, 7.0, and 7.5), and in no case were thiols consumed above background levels when flavonoids were used as substrates. Flavonoids alone did not react with the Ellman thiol detection reagent. We conclude that, in vitro, no conjugation reaction takes place.

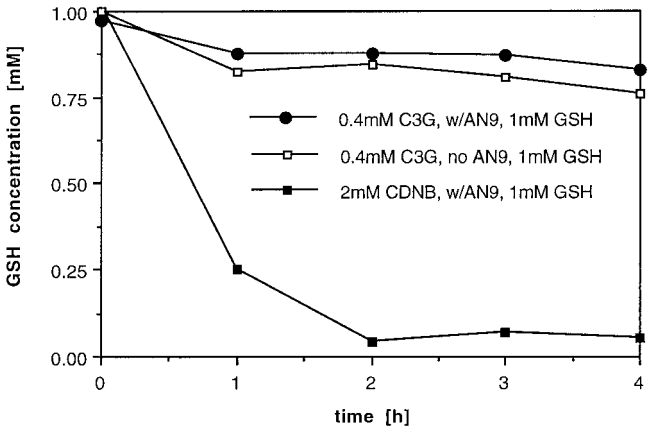


**Figure 3.** Measurement of thiol concentration during enzymatic incubations. To determine if conjugates do form that are unstable or acid-labile, and hence could not be detected by the HPLC assay used (Fig. 2), AN9 was incubated with GSH and C3G. The concentration of free thiol-group of GSH was determined initially and then every hour using the colorimetric Ellman reaction (see "Materials and Methods"). When no enzyme is added to the incubation (□), a slight reduction in thiol concentration can be detected, probably resulting from GSH oxidation. When enzyme is used in the incubation (●), the reduction in thiol concentration is not significantly different from the enzyme-free assay, demonstrating that AN9 does not catalyze conjugate formation. When CDNB is used as a substrate, thiol concentration decreases sharply (■).

### Absence of Conjugate in Vivo

Conditions used in the in vitro assays may not reflect the in vivo situation. Previously, feeding experiments have been used effectively in experiments establishing the order of action of enzymes in the anthocyanin pathway (McCormick, 1978). Thus we adapted this method to investigate whether conjugates exist in vivo by incubating sections of colored and white petunia petals with [$^3$H]UDP-Glc, [$^{35}$S]GSH, and naringenin, a precursor in anthocyanin biosynthesis. In the colored petals enzymes convert chalcone to anthocyanin, but in white tissues no such conversion should occur. HPLC was used to analyze the extracts. Peaks absorbing at 510 nm or 350 nm and carrying $^3$H and $^{35}$S label would be expected for an anthocyanin conjugate with GSH. After an overnight incubation $^3$H-glucosylated anthocyanin was readily detected in the colored tissue, although only roughly 1% of the $^3$H label taken up by the cell was contained in this peak at 13 min. In some experiments several other small $^3$H peaks could be detected. The $^{35}$S-elution trace exhibited large peaks near the flow-through, corresponding to GSH and GSSG, and sometimes a noisy baseline with high background between 10 and 15 min, but with no clearly resolved peaks. This could indicate some form of unspecific association of sulfur containing materials with hydrophobic compounds. We detected no specific, covalent modification of a flavonoid. Except near the flow-through, none of the $^3$H and $^{35}$S peaks co-eluted. We conclude that at least the flavonoid glycones do not form detectable levels of conjugates with GSH in vivo.

Because the intracellular GSH concentration is high, it is difficult to assess if a conjugate could be detected by the addition of a small quantity of labeled GSH (Marrs et al., 1995). To address this problem we used large quantities of $^{35}SO_4^{2-}$ to saturate petals with $^{35}$S. Using a similar protocol as before

$^{35}SO_4^{2-}$ and naringenin were supplied to colored petals. We detected no $^{35}S$-label in the anthocyanin and flavonoid peaks (data not shown). This experiment confirms that tissue-synthesizing anthocyanin accumulates no detectable $^{35}S$-labeled product.

### Binding of Flavonoids to AN9

If AN9 is required for anthocyanin sequestration in vivo, but does not form a conjugate, we hypothesized that AN9 could be a flavonoid-binding protein. Several assays were used to determine if AN9 could bind flavonoids. The first assay used was the inhibition of CDNB-conjugating activity (Droog et al., 1993) by flavonoids (Table I). The effect of flavonoids on other GSTs (maize GSTI, maize GSTIII, and equine GST) was also measured. Conjugation of CDNB by AN9 was strongly inhibited or competed by all flavonoids except luteolin 7-glucoside and naringenin (Table I; Fig. 1). Naringenin is a colorless substrate that has unconjugated carbon bonds in the C-ring, resulting in a non-planar structure; all other flavonoids examined are planar molecules. Of the substrates tested the planar substrates, therefore, had a higher affinity for the binding site. Luteolin 7-glucoside carries a bulky group on the A-ring, indicating that this part of the molecule is also important for binding. Conversely, because luteolin 4′-glucoside was as strong an inhibitor as luteolin, we conclude that the B-ring is of minor importance for binding. C3G, which has a Glc substitution on the C-ring, inhibited CDNB-conjugating activity, but not as strongly as the aglycones tested. The compounds abscisic acid, indole acetic acid, Glc, Trp, Gln, and Pro (75 μM) did not inhibit the CDNB conjugating activity of AN9 (data not shown).

Equine GST was not inhibited by flavonoids, but GSTIII, which can complement *bz2* tissue in a particle gun assay (Alfenito et al., 1998), exhibited similar inhibition parameters to those of AN9. GSTI was intermediate between AN9 and equine GST in terms of inhibition by flavonoids.

As an independent measure of flavonoid interaction with GSTs, equilibrium dialysis was performed using [$^3$H]isoquercitrin and [$^3$H]luteolin (Fig. 4). It is surprising that all GSTs tested bound both luteolin and isoquercitrin (Table II); even the equine GST that showed no inhibition by flavonoids exhibited some flavonoid binding in this assay.

We found that several molecules of non-substrate ligand were bound per GST dimer. Each GST dimer is normally thought to comprise two binding sites for hydrophobic molecules (H-sites) and two GSH bind-

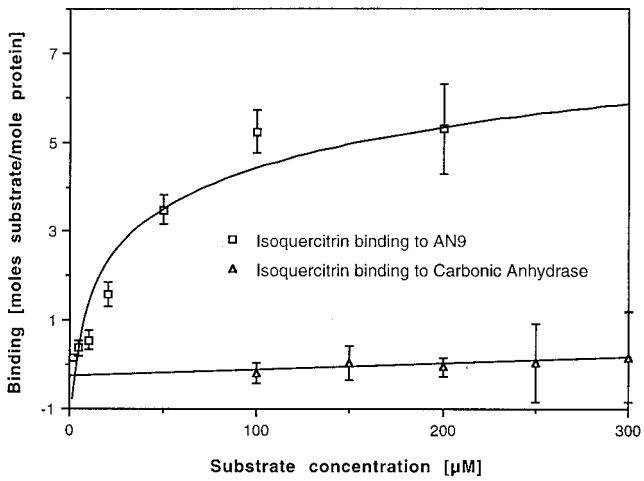


**Figure 4.** Equilibrium dialysis. Two data sets are shown. Binding of isoquercitrin by AN9 (□) and binding of isoquercitrin by carbonic anhydrase (△) are shown. The substrate concentration is plotted against number of molecules bound. Bars indicate SE from a single experiment performed in triplicates. Curves such as these were converted to Lineweaver-Burk plots used to calculate the $K_d$* and $b_{max}$ values given in Table II.

**Table I.** *Inhibition of GSTs by flavonoids ($C_{50}$ values in μM)*

Inhibition of the CDNB conjugating activity of AN9, GSTI, GSTIII, and horse liver GST (eGST) by flavonoid substrates. The $C_{50}$ values (in μM were graphically determined by plotting activity in function of inhibitor concentration. Both AN9 and GSTIII are strongly inhibited by most flavonoids, whereas GSTI is inhibited to a lesser degree. Equine GST is not inhibited by flavonoids. Ethacrynic acid was included as a non-flavonoid inhibitor of CDNB activity.

| | AN9 | GSTI | GSTIII | eGST |
|---|---|---|---|---|
| Quercetin | 10–25 | >300 | 10–25 | NI[a] |
| Isoquercitrin | 25 | 200–250 | 10–25 | NI |
| Cyanidin | 5 | NI | 50–100 | NI |
| C3G | 50 | NI | 50–100 | NI |
| Luteolin | 5 | 50–100 | 50–100 | NI |
| Luteolin-4′-Glc | 5 | 50 | 5–10 | NI |
| Luteolin-7-Glc | >200 | >200 | 25–50 | NI |
| Naringenin | 200–250 | NI | 25 | NI |
| Ethacrynic acid | 5 | 25 | 5 | 50 |

[a] NI, No inhibition.

**Table II.** *Equilibrium dialysis-binding data*

Equilibrium dialysis-binding data. All GSTs tested bound flavonoids to a certain extent, even those that are not inhibited in their CDNB conjugating activity by flavonoids. AN9 had the highest affinity for isoquercitrin of all GSTs tested. Values are given in μM for the $K_d$* values, and in numbers of molecules bound per mole of protein molecule for the $b_{max}$ values. The constants were determined from Lineweaver-Burk plots. The AN9 data represent the means of two experiments performed in triplicate; for the other enzymes, data are shown from one experiment performed in triplicate.

| Protein | Substrate | $K_d$* | $b_{max}$ |
|---|---|---|---|
| | | μM | |
| AN9 | Isoquercitrin | 88 | 7.2 |
| AN9 | Isoquercitrin + GSH | 66 | 4 |
| AN9 | Luteolin | 185 | 11.7 |
| AN9 | Luteolin + GSH | 121 | 9.6 |
| GSTIII | Isoquercitrin + GSH | 157 | 10.6 |
| GSTIII | Luteolin + GSH | 215 | 13.9 |
| GSTI | Isoquercitrin + GSH | 268 | 8.75 |
| GSTI | Luteolin + GSH | 142 | 15.3 |

ing sites (G-sites; Reinemeier et al., 1996). For isoquercitrin, binding without the addition of GSH resulted in about eight molecules of isoquercitrin being bound to each enzyme molecule, with an apparent $K_d$* of 90 $\mu$M. In assays that contained GSH four molecules were bound per GST dimer, and the $K_d$* was 66 $\mu$M. The fact that some enzymes were not inhibited by flavonoids, but readily bound them in the equilibrium dialysis assay suggests that multiple flavonoid-binding sites exist on GSTs. AN9 had the highest affinity for isoquercitrin among the enzymes tested. Bovine serum albumin (BSA) also bound multiple flavonoid moieties per molecule. Binding of flavonoids to BSA has been previously reported (Boulton et al., 1998). In fact it has been speculated that a primary role of BSA is the binding of dietary phytochemicals such as flavonoids (Baker, 1998). Carbonic anhydrase exhibited very little flavonoid-binding capacity compared with either GSTs or BSA and served as negative control (Table II).

To further assess binding of flavonoids to AN9 we analyzed quenching of intrinsic protein fluorescence by flavonoid substrates. AN9 has only three Trp residues, and all are located in the carboxy-terminal one-half of the protein. It is surprising that luteolin and cyanidin quenched fluorescence very strongly; at a concentration of only 1 $\mu$M, fluorescence was quenched by about 30%. This result indicates that binding occurs either near the Trp residues, or that a conformational change is induced in the AN9 molecule, which changes the chemical environment of the Trp residues. C3G and isoquercitrin did not quench Trp fluorescence as strongly as did the aglycones; at 1 $\mu$M, quenching was about 10%. This result indicates that the binding sites for C3G are either more distant from the Trp residues or a different conformational shift occurs.

### Does Substrate Binding Induce a Conformational Change in AN9?

When GSTs bind substrates, they undergo conformational changes as evidenced by structural analysis using x-ray crystallography (Neuefeind et al., 1997). Using spectral analysis with circular dichroism, we could not detect major conformational changes in AN9 upon binding of flavonoids and GSH (data not shown). We did find, however, that mobility on native polyacrylamide gels was affected when recombinant AN9 was pre-incubated with flavonoids (data not shown), which could indicate a conformational change. Resolution of possible conformational changes in AN9 after substrate binding will likely require the precision of x-ray crystallographic examination.

## DISCUSSION

AN9 is required for efficient vacuolar sequestration of anthocyanin in petunia. Like the *Bz2* gene of maize *An9* encodes a GST based on its ability to catalyze conjugation of GSH to CDNB. Despite the parallels between anthocyanin biosynthesis and xenobiotic detoxification, AN9 does not appear to conjugate GSH to anthocyanin or other flavonoids in vitro (Fig. 2). Similarly, in measuring thiol concentration during incubations, we found rapid disappearance of GSH when CDNB was the substrate, whereas free GSH remained nearly constant in reaction mixtures containing enzyme, flavonoids, and GSH (Fig. 3). Novel HPLC peaks and thiol consumption could be accounted for by the spontaneous oxidation of GSH to GSSG.

Inhibition of animal GSTs by flavonoids has also been studied (Merlos et al., 1991). To our knowledge no conjugated flavonoids have ever been described from such studies. The only flavonoid-derived conjugate with GSH reported is that with the phytoalexin medicarpin (Li et al., 1997). However, the medicarpin structure could undergo a ring-opening reaction in the heterocyclic ring that is absent from anthocyanins and related molecules.

The existence of anthocyanin-glutathione conjugates was inferred from feeding radiolabeled GSH to Black Mexican Sweet corn protoplasts after electroporation with the transcription factors *R* and *C* to induce anthocyanin synthesis. Extracts of these cells were then analyzed by two-dimensional TLC. The radiolabel colocalized with anthocyanin pigments on the TLC plates, but no further attempt was made to characterize these putative conjugates (Marrs et al., 1995). Colocalization of radiolabel does not prove the existence of anthocyanin conjugates, and under the TLC conditions employed, many cellular constituents colocalize with anthocyanin pigments (L. Mueller, unpublished data).

Others have published protocols for producing the C3G-glutathione conjugates using nonenzymatic, chemically quite severe conditions (borate buffer, pH 9, 55°C, overnight with [$^3$H]GSH; Lu et al., 1998). In our hands HPLC analysis of such reaction mixtures spiked with [$^{35}$S]GSH failed to detect conjugates, although oxidized GSSG was formed. GSSG formation occurred to the same extent when the flavonoids were omitted from the reaction. We suspect that this method produced mainly GSSG, which is competent for import in the ATP-binding cassette (ABC)-transporter assay with resealed vacuoles (Lu et al., 1998); the trace quantities of radiolabeled material taken up during the in vitro assay for pump activity preclude chemical analysis of the product. A secondary problem with the original chemical protocol is that at high pH, tritium label can be exchanged and hence tritium can potentially label other hydrogen-containing compounds with no concomitant formation of conjugates. This problem is largely avoided when [$^{35}$S]GSH is used.

To test if GSH conjugates of anthocyanins occur in vivo we incubated petunia petals with naringenin,

[$^3$H]UDP-Glc, and [$^{35}$S]GSH or $^{35}SO_4^{2-}$. HPLC analysis of extracts revealed that [$^3$H]Glc was incorporated in anthocyanin pigments, but no $^{35}$S-sulfur label was found associated with these peaks, indicating that anthocyanins do not form GSH conjugates in vivo. These findings are confirmed in the literature; of the hundreds of new flavonoid structures described in the past seven years (Harborne and Williams, 1995, 1998), none contained GSH. In addition considering the structure of anthocyanins, it is not evident where GSH conjugation could occur. Unlike CDNB, anthocyanins contain no strong electrophilic centers, making it difficult to conceive how a stable conjugate could form. To date, all proven GST substrates exhibit sufficient electrophilicity to undergo spontaneous conjugation with GSH, and GSH conjugates of compounds like CDNB can be synthesized easily (Coleman et al., 1997). Conjugates of certain herbicides are readily detected in vivo (Wolf et al., 1996; Coleman, 1997). The GSH tag is slowly degraded in the vacuole by carboxypeptidases, yielding a population of conjugates carrying GS-, a γ-glutamyl-Cys moiety, or Cys (Wolf et al., 1996). Experiments with monochlorobimane, which fluoresces when conjugated to GSH, demonstrate that the sulfur bond is stable (Coleman et al., 1997). If the processes of translocation through the tonoplast were identical for xenobiotic herbicides and anthocyanins, it should be possible to observe both the initial GSH conjugate and the derivative metabolites in the vacuole.

If no conjugates form we must consider why GSTs are required for anthocyanin sequestration. We assayed whether AN9 can bind anthocyanins and related flavonoids to test the idea that AN9 potentially acts as a carrier protein. Three lines of evidence support the notion that AN9 does interact with and bind flavonoids: (a) Flavonoids strongly inhibit the CDNB conjugating activity of AN9 (Table I); (b) selected flavonoids bind to AN9 in an equilibrium dialysis assay (Fig. 4; Table II); and (c) flavonoids exhibit strong quenching of Trp fluorescence of AN9. It is well established that GSTs do act as binding proteins in animals. GSTs have a high affinity for specific bile acids, bromosulfophthalein, fatty acids, bilirubin, and certain drugs (Hayes and Pulford, 1995), but the GSTs involved do not form GSH conjugates with their substrates (Litwack et al., 1971; Hayes and Pulford, 1995). These GSTs have been termed "ligandins" and the nonenzymatic substrates are referred to as "non-substrate ligands."

The precise functions of ligandin GST binding to non-substrate ligands remain unclear (Hayes and Pulford, 1995). Ligandins have one high-affinity site per dimer for their non-substrate ligand. In one report human GSTs α, β, γ, δ, and ε have $K_d$ values of 65, 110, 34, 18, and 34 μM for their ligands (Kamisaka et al., 1975). The calculated $K_d$* values for luteolin and isoquercitrin interacting with AN9, when assayed with equilibrium dialysis, were similar and ranged from 50 to 100 μM. The $K_d$* values we obtained using equilibrium dialysis could be overestimated as a result of loss of binding capacity of the enzyme over the 24-h assay. During the incubation time, however, enzymatic activity did not decrease significantly when measured with CDNB (data not shown). AN9 bound four molecules of isoquercitrin per dimer in the presence of GSH; these may include both high and low affinity sites.

What is the function of the simultaneous binding of flavonoid substrate and GSH if no free conjugate is formed? We cannot exclude the possibility that local conditions are such that the flavonoid-GSH conjugates are formed on the enzyme, but such conjugates are not stable in solution. Maleylacetoacetate isomerase is a GST acting in the Tyr catabolic pathway in animals (FernandezCanon et al., 1999), catalyzing a cis/trans isomerization. Transient GSH conjugates must form on the enzyme during isomerization, but such intermediates cannot be recovered (Fernandez-Canon et al., 1999). In the case of anthocyanin, however, the structure of the cytosolic and vacuolar forms are identical, ruling out a GST-mediated isomerization step in the biosynthetic pathway. Our working model is that C3G binding to a GST prevents oxidation of the flavonoid molecule; the defining characteristic of *bz2* mutants is accumulation of oxidized and cross-linked C3G in the cytosol (Alfenito et al., 1998). In addition, because flavonoids are also cytotoxic and genotoxic compounds that can oxidize protein and intercalate into DNA, it is possible that an escort protein is required during anthocyanin synthesis to prevent cellular damage (Ahmed et al., 1994). Future experiments will address a third possible function, which is the possibility that GSTs facilitate delivery of their flavonoid cargo to specific cellular compartments.

Several plant GSTs with ligandin-like properties have been described. These include GSTs with the capacity to bind auxin (Bilang et al., 1993; Zettl et al., 1994; Bilang and Sturm, 1995; Watahiki et al., 1995) and cytokinin (Gonneau et al., 1998). The GST isolated from *Hyoscyamus muticus* (*Hmgst-1*) by photoaffinity labeling with 5-azido-indole 3-acetic acid showed only weak inhibition in a noncompetitive manner by indole acetic acid in the standard CDNB assay, and no auxin-glutathione conjugates were found (Bilang et al., 1993; Bilang and Sturm, 1995). Unfortunately these authors never reported equilibrium dialysis or other similar procedures to determine the $K_d$ of auxin for HMGST-1, making the assessment of the biological significance of this binding difficult. The functions of auxin-binding GSTs are unknown. Similarly, a radiolabeled azido-cytokinin was bound specifically by a GST prepared from tobacco (*Nicotiana plumbaginifolia*) (Gonneau et al., 1998). In light of our results it is possible that individual GSTs could function as binding proteins for specific compounds in the cell, without conjugate

formation. This function is consistent with the relatively high concentration of GSTs in the cell. GSTs that bind specific hormones could modulate effective hormone concentration through their binding activity or by mediating sequestration to other intracellular compartments, as in the case of anthocyanins.

A GST from Arabidopsis isolated by photo-affinity labeling with auxin copurified with the plasma membrane (Zettl et al., 1994). BZ2 also partially sediments in the membrane fraction (C. Pairoba and L. Mueller, unpublished observations). Membrane association could reflect an interaction between GSTs and specific membrane proteins. Indeed if GSTs are escorts involved in intracellular metabolite movement, we could expect them to interact with membrane transport proteins such as ABC-pumps (Martinoia et al., 1993) that have been implicated in maize anthocyanin sequestration (Marrs et al., 1995).

Without a GSH conjugate, how can anthocyanins be transported into the vacuole by glutathione-conjugate dependent (GS-X) pumps? A likely model is provided by vincristine transport in the liver. In this case the formation of a GSH conjugate is apparently not required; the transport is accomplished by a cotransport mechanism with reduced GSH (Loe et al., 1998). A similar mechanism may be at work for anthocyanins.

Other mechanisms for anthocyanin import to vacuoles have been proposed. Klein et al. (1996) have observed that unmodified flavonoid glucosides were readily imported into isolated barley vacuoles. The import of the flavonoid glucoside was compared with a herbicide glucoside and found to require different energizing mechanisms (Klein et al., 1996). Transport of the flavonoid glucoside was ΔpH dependent, whereas the herbicide glucoside was transported by an ABC transporter. Moreover, anthocyanins from maize are mono- and di-acylated with malonyl residues (Harborne and Self, 1986). This acylation has been linked to vacuolar import of anthocyanins (Hopp and Seitz, 1987) and other flavonoids (Matern et al., 1986) in in vitro assays that employed radiolabeled substrates and isolated vacuoles from a carrot cell line. De-acylated flavonoids were not efficiently taken up, whereas acylated forms were readily imported. In these experiments no addition of GSH or GST was necessary for sequestration of acylated flavonoids. It is possible that different plants utilize different import mechanisms for the vacuolar sequestration of anthocyanins; the acylated anthocyanin used in the carrot experiment had been isolated from a carrot cell culture, and only vacuoles prepared from the carrot line readily imported the anthocyanin. Vacuoles prepared from other plant species did not import the acylated anthocyanin (Hopp and Seitz, 1987). A concern with these experiments is that the in vitro assay may not reflect in vivo conditions, as the pH optimum of import was in the range of pH 7.5 to 8.0, and little import was observed at the normal cytoplasmic pH of 7.0.

Plant genomes encode dozens of GSTs; to date there are more than 30 GST-like sequences in the Arabidopsis database. With the exception of the GSTs required for vacuolar sequestration of anthocyanin, the specific in vivo functions of individual plant GSTs remain unknown. We speculate that many could serve as binding and carrier proteins for specific endogenous compounds.

## MATERIALS AND METHODS

### Reagents

Flavonoids and anthocyanins were purchased from Extrasynthese (Lyon, France), or from Indofine Chemical Corporation (Somerville, New Jersey). All other chemicals were obtained as reagent grade from Sigma (St. Louis). [$^3$H]UDP-Glc was obtained from NEN (Boston). [$^3$H]Luteolin was a gift from Sharon Long (Department of Biological Sciences, Stanford University, Stanford, CA).

### Purification of AN9, GSTI, and GSTIII

AN9 was expressed as a 6 × HIS-tagged fusion protein in *Escherichia coli* using a previously described construct (Alfenito et al., 1998). Maize GSTI and maize GSTIII cDNAs were amplified by PCR and cloned into a pQE30 expression vector (Qiagen, Düsseldorf, Germany), yielding the constructs pQEGSTI and pQEGSTIII, and expressed as 6 × HIS-tagged fusion proteins. Constructs were transformed into *E. coli* strain JM105. Cells were grown overnight at 37°C with shaking in 3 mL of Luria-Bertani media containing 50 μg/mL carbenicillin and then diluted 1:500 in 1 L of the same medium. Growth was monitored by measuring turbidity at 600 nm; 0.2 mM isopropylthio-β-galactoside was added when turbidity reached 0.5 absorption unit. Incubation was continued for 4 h. The cells were collected by centrifugation at 10,000*g*, then resuspended in 5 mL of extraction buffer (0.1 M phosphate buffer, pH 8.0, 20 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride, and 10% [v/v] glycerol) containing 1 mg mL$^{-1}$ lysozyme, and incubated on ice for 1 h. All subsequent steps were performed at 4°C. To ensure complete cell lysis the resuspended material was drawn into a syringe through a 19-gauge needle several times until viscosity was reduced; the cell lysate was collected into Eppendorf tubes. DNA was precipitated by adding polyethyleneimine to a final concentration of 0.1% (w/v; 10% stock prepared from the commercially available 50% stock) and removed by centrifugation at full speed in a microcentrifuge. After decanting the supernatant, the centrifugation step was repeated. The HiTrap Chelating cartridge (1-mL volume, Pharmacia, Piscataway, NJ) used for protein purification was equilibrated with 100 mM $NiCl_2$ solution and extensively washed with water and extraction buffer. Using a 10-mL syringe, the protein-containing supernatant was loaded onto the cartridge and washed with 5 mL of extraction buffer. The absorbed protein eluted with 5 mL of extraction buffer containing 250 mM

imidazole. One-milliliter fractions were collected throughout the procedure. The protein-containing fractions were identified using a dye-binding assay (Bradford, 1976); these fractions were pooled and dialyzed against 50 mM phosphate buffer and pH 6.8, 20% (v/v) glycerol, with several changes of buffer. Glycerol was added to a final concentration of 40% to prevent freezing during storage at −20°C.

### Determination of Thiol Concentration

To determine whether GSH conjugates of flavonoids form, the concentration of the free thiol group of GSH was measured using the Ellman reagent, 5–5′-dithio-bis (2-nitrobenzoic acid). A 5 mM solution was prepared in 0.1 M phosphate buffer containing 0.1 mM EDTA, pH 7.2. Reactions were set up in 96-well plates with 40 μL of 0.2 M phosphate buffer, pH 6.8, 1 mM GSH, and 20 μL of enzyme or a 40% (v/v) glycerol solution (mock treatment). Flavonoid was added to a final concentration of 400 μM from a 10 mM stock solution prepared in ethanol. CDNB was added to a final concentration of 2 mM from a 100 mM stock solution in ethanol. For every time point, a separate reaction was set up and thiol concentration was monitored by adding an equal volume (60 μL) of Ellman's reagent at the appropriate time. Absorbance was measured immediately after addition of reagent at 412 nm in a plate reader (Bio-Rad, Richmond, CA). A standard curve was prepared using GSH at concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM.

### Inhibition of the CDNB Enzymatic Reaction

GST activity toward CDNB was measured by adapting the procedure described by Holt et al. (1995). Assay conditions were 0.1 M phosphate buffer, pH 6.8, and 1 mM GSH (diluted from a 50 mM stock). CDNB was added from a 100 mM stock prepared in ethanol to a final concentration of 1 mM just before measurement. Absorption was measured at 340 nm after 1 to 5 min, as appropriate, for maize GSTI and maize GSTIII, and after 30 min for AN9. All measurements were adjusted by subtracting nonenzymatic conjugation of CDNB (Coleman, 1997). Inhibitors were added from 10 or 1 mM stock solutions in ethanol to achieve 1 to 200 μM inhibitor concentration; inhibition was calculated by comparing the resulting activity relative to the activity without inhibitor.

### Production of [$^3$H]Isoquercitrin

[$^3$H]Isoquercitrin was produced enzymatically using a crude protein extract of colored corn husks; this extract contains the BZ1 enzyme, a flavonoid glucosyl transferase. The extract was incubated with quercetin and [$^3$H]UDP-Glc as substrates (Raboy et al., 1989). For production synthesis, a total volume of 200 μL was used.

### HPLC Analysis of Pigments

Extracts were analyzed on a DX-500 HPLC system (Dionex, Sunnyvale, CA) fitted with a UV-VIS detector (AD20, Dionex). A Radiomatic 150TR (Packard, Meriden, CT) radiodetector was used with Ultima Flow M scintillation fluid (Packard) at a flowrate of 3 mL min$^{-1}$. A reverse phase C18 column was used for flavonoid and DNP-GS analysis (Rainin, Walnut Creek, CA). Phase A was 1% (v/v) acetic acid in water and phase B was 100% (v/v) acetonitrile. The flowrate was set to 1 mL min$^{-1}$. The column was equilibrated with 97% A, 3% B. After injection the column was washed for 2 min with the same elution conditions, and then a gradient was run to 50% phase B in 17 min. A steeper gradient was then run to 100% B in 2 min. Phase B was brought back to 3% in 3 min, and the column washed for 6 min.

### Synthesis and Analysis of DNP-GS

CDNB (1 mM), GSH (1 mM), and 0.2 μL of [$^{35}$S]GSH were incubated overnight in 100 μL of 0.1 M borate buffer, pH 9.0. For HPLC analysis 10 μL of the preparation was diluted to 500 μL with 1% (v/v) acetic acid. The diluent was directly injected into the HPLC system and analyzed using the same protocol as for the analysis of the pigments described in the previous section.

### Equilibrium Dialysis

Equilibrium dialysis was performed in 1-mL modules obtained from Fisher (Pittsburgh). The modules were fitted with Spectra/Por 7 dialysis tubing with a molecular mass cutoff of 25,000 daltons (Spectrum, Houston). It is interesting that lower cutoff membranes did not allow efficient equilibration of flavonoids, probably as a result of intermolecular associations between the molecules. An incubation time of 24 h at 4°C permitted a 100 μM isoquercitrin solution added to one side of the membrane to equilibrate to 95%. Protein was present on one side at a concentration of 3 to 12 μM. Flavonoid was added to a concentration of 2, 5, 10, 20, 50, 100, or 200 μM on both sides, as well as 0.1 M phosphate buffer and 2 mM GSH; $^3$H-labeled flavonoid (60,000 cpm) was added to the non-protein-containing side. The volume on both sides was 100 μL. After overnight incubation at 4°C, triplicate samples of 20 μL were measured in a scintillation counter (1 mL of scintillation fluid was added; EcoLite, ICN, Costa Mesa, CA). The protein concentration was measured at the end of the dialysis period (Bradford, 1976). No protein was detected on the non-protein-containing side. The difference in counts between the two chambers was divided by the number of counts in the protein-containing chamber, and this value was multiplied by the nominal substrate concentration. This number was then divided by the protein concentration expressed in micromoles to obtain the number of moles substrate bound per mole protein. Results were plotted in Lineweaver-Burk formats, and a global binding constant, $K_d$*, and the number of molecules bound, $b_{max}$, were determined.

### Trp Fluorescence Quenching

Fluorescence quenching was measured in 3-mL quartz cuvettes containing 2 mL of liquid, in a luminescence spec-

trometer (LS50B, Perkin Elmer, Norwalk, CT). The excitation was set to 280 nm, and the region between 300 and 400 nm was scanned for emission. Emission maxima and intensity levels were determined using the built-in software. Enzymes were diluted to a 2 $\mu$M concentration in 0.1 M phosphate buffer, pH 6.8. Flavonoids were added from 10 and 1 mM stock solutions, at final concentrations of 0.25, 0.5, 0.75, 1, 5, and 10 $\mu$M. GSH was added from a 50 mM stock solution to a final concentration of 1 mM.

### In Vivo Assays for Anthocyanin Glycosides and GSH Conjugates

In a colabeling experiment sections of young colored or white petunia (*Petunia hybrida*) petals (0.5 $cm^2$) were incubated overnight with [$^3$H]UDP-Glc (1 $\mu$L, $6 \times 10^5$ cpm) and [$^{35}$S]GSH (1 $\mu$L, $3 \times 10^6$ cpm) in 200 $\mu$L of distilled water. To eliminate radioactivity that had not been taken up by the petals, the water was removed, and 200 $\mu$L of extraction solution added (1% [v/v] acetic acid). The pigments were extracted by grinding with a small plastic pestle in an Eppendorf tube. The liquid phase was transferred to a fresh Eppendorf tube and was centrifuged at full speed in a microcentrifuge. At this stage, about 30% of the initial radioactivity for each isotope was present in the supernatant, roughly corresponding to radioactivity that had been taken up by the petals. The supernatant was appropriate for direct analysis using HPLC.

In a second set of experiments petals were incubated with $^{35}SO_4^{2-}$ (100 $\mu$L) overnight. The petals were then washed three times with 300 $\mu$L of water, and ground in 200 $\mu$L of 1% (v/v) acetic acid. After centrifugation, the supernatant was analyzed on HPLC as in the previous experiment.

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