*Journal of Experimental Botany, Vol. 59, No. 13, pp. 3621–3634, 2008*
doi:10.1093/jxb/ern217
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

**RESEARCH PAPER**

# Purification, molecular cloning, and characterization of glutathione *S*-transferases (GSTs) from pigmented *Vitis vinifera* L. cell suspension cultures as putative anthocyanin transport proteins

**Simon Conn[1,2], Chris Curtin[1,3], Annie Bézier[4], Chris Franco[1] and Wei Zhang[1,*]**

[1] *Department of Medical Biotechnology, Flinders University, Adelaide, Australia, 5042*

[2] *School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, Urrbrae, Australia, 5064*

[3] *Australian Wine Research Institute, Waite Campus, Urrbrae, Australia, 5064*

[4] *Laboratoire de Biologie et Physiologie Végétales, Equipe de Biochimie et Biologie Moléculaire des Plantes, Université de Reims Champagne-Ardenne, 51687 Reims cedex 2, France*

Received 27 May 2008; Revised 1 July 2008; Accepted 24 July 2008

## Abstract

**The ligandin activity of specific glutathione *S*-transferases (GSTs) is necessary for the transport of anthocyanins from the cytosol to the plant vacuole. Five GSTs were purified from *Vitis vinifera* L. cv. Gamay Fréaux cell suspension cultures by glutathione affinity chromatography. These proteins underwent Edman sequencing and mass spectrometry fingerprinting, with the resultant fragments aligned with predicted GSTs within public databases. The corresponding coding sequences were cloned, with heterologous expression in *Escherichia coli* used to confirm GST activity. Transcriptional profiling of these candidate GST genes and key anthocyanin biosynthetic pathway genes (*PAL*, *CHS*, *DFR*, and *UFGT*) in cell suspensions and grape berries against anthocyanin accumulation demonstrated strong positive correlation with two sequences, *VvGST1* and *VvGST4*, respectively. The ability of VvGST1 and VvGST4 to transport anthocyanins was confirmed in the heterologous maize *bronze-2* complementation model, providing further evidence for their function as anthocyanin transport proteins in grape cells. Furthermore, the differential induction of VvGST1 and VvGST4 in suspension cells and grape berries suggests functional differences between these two proteins. Further investigation of these candidate ligandins may identify a mechanism for manipulating anthocyanin accumulation *in planta* and *in vitro* suspension cells.**

Key words: Anthocyanin transport, glutathione *S*-transferase, ligandin, plant cell culture, *Vitis vinifera*.

## Introduction

Plants contain a variety of glutathione-binding proteins, including glutathione lyases, peroxidases, reductases, and *S*-transferases (GSTs) (Marrs, 1996). The GSTs are unique in this group as they are all soluble or loosely membrane-associated dimers with a monomeric size of 15–28 kDa, and together comprise 1–3.5% of total cellular protein (Droog *et al*., 1995; Pairoba and Walbot, 2003). GSTs catalyse the conjugation of the tripeptide glutathione (γ-Glu-Cys-Gly; GSH) to a variety of electrophilic compounds to direct them to specific sites both intra- and extracellularly. In mammals, GST subunits are classified into α, μ, π, σ, and θ classes on the basis of amino acid identity, immuno-cross-reactivity, and substrate specificity (Buetler and Eaton, 1992; Mannervik *et al*., 1992). The majority of plant GSTs are most similar to the θ class, and are further classified into three phylogenetic types based on sequence similarity and intron/exon structure (Droog *et al*., 1993; Droog, 1997). Type I GSTs, with two introns, have been identified in numerous plant species. Type II GSTs

* To whom correspondence should be addressed. E-mail: wei.zhang@flinders.edu.au

have been identified in wheat and carnation, with the gene in carnation containing nine introns (McGonigle *et al*., 2000). Type III GSTs contain a single intron and make up the second largest family of plant GSTs, including many of the GSTs originally identified as auxin-regulated proteins (Takahashi *et al*., 1995; Edwards and Dixon, 2005).

GSTs, originally characterized by their ability to modify xenobiotics covalently by glutathionation, are rapidly induced by their substrates—in particular plant herbicides (Marrs *et al*., 1995; Aharoni *et al*., 2002; Dean *et al*., 2005). In addition, GSTs act as non-enzymatic carrier proteins (ligandins) enabling intracellular shuttling of endogenous compounds in animals, plants, and prokaryotes. Within plants, these ligands include indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), labile cytochrome precursors, and the ubiquitous plant pigments, anthocyanins (Jones, 1994; Bilang and Sturm, 1995; Marrs *et al*., 1995; Mueller *et al*., 2000; Lederer and Böger, 2005). The soybean GH2/4 gene is inducible not only by strong auxins (2,4-D; 2,4,5-T; α-NAA) and salicylic acid (SA), but also by numerous other electrophilic compounds, including weak auxins (β-NAA), inactive auxin analogues (2,3-D and 2,3,6-T), and inactive SA analogues (3-hydroxybenzoic acid, 4-hydroxybenzoic acid) (Ulmasov *et al*., 1994, 1995). Furthermore, the Hmgst-1 protein from *Hyoscyamus muticus* able to bind IAA exhibits an expression profile that correlates with accumulation of IAA, suggesting that this GST is involved in intracellular IAA transport (Bilang *et al*., 1993; Bilang and Sturm, 1995). Thus, it is clear that different auxins and potentially other ligands induce and bind GSTs differently. Beyond this work on auxins, few studies relate GST transcription or expression with accumulation of endogenous substrates. The well-characterized anthocyanin biosynthetic pathway in grapevines thus provides an ideal target for analysing transcriptional correlation of GSTs with another family of non-substrate ligands.

The state of protonation influences the colour of anthocyanins, and in many plant species they are concentrated within structures known as anthocyanic vacuolar inclusions, within the acidic central vacuole (Pecket and Small, 1980; Conn *et al*., 2003). Their synthesis, however, has been demonstrated to occur predominantly in the cytosol. Indeed for *Arabidopsis*, the anthocyanin biosynthetic complex has been localized to the cytosolic face of the rough endoplasmic reticulum (reviewed in Winkel, 2004). Furthermore, the localization of an anthocyanin methyltransferase from *Petunia hybrida* (Jonsson *et al*., 1983) and anthocyanin acyltransferases from *Gentiana triflora* (Fujiwara *et al*., 1998) and *Perilla frutescens* (Yonekura-Sakakibara *et al*., 2000) to the cytosol suggests that complete anthocyanins, rather than intermediates, are transported into the vacuole.

Gene knockout and complementation studies have demonstrated that GSTs are indelibly involved in anthocyanin transport. A *Zea mays* knockout mutant of a single GST [*Bronze-2* (*Bz2*)] accumulated anthocyanins in the cytosol only, at concentrations <10% of those found in wild-type lines (Mueller and Walbot, 2001; Goodman *et al*., 2004). Anthocyanin-transporting GSTs have been identified by large-scale mutagenesis screens in other species, including petunia (*An9*; Alfenito *et al*., 1998), carnation (*Fl3*; Larsen *et al*., 2003), and *Arabidopsis* (*TT19*; Kitamura *et al*., 2004). Kitamura *et al*. (2004) demonstrated that TT19 played an additional role in seed deposition of proanthocyanidins (tannins). While binding of both Bz2 and An9 to anthocyanins has not been directly shown, various studies have inferred this interaction by the use of equilibrium dialysis and tryptophan quenching with the recombinant proteins (Marrs *et al*., 1995; Mueller *et al*., 2000). *Bz2* knockout (*bz2*) suspension cells, when complemented with *Bz2* and exposed to [$^{35}$S]GSH, produced $^{35}$S-labelled compounds that co-migrated with anthocyanins when separated by thin-layer chromatography. While this is suggestive of covalent glutathionation *in vivo*, no further analysis was performed on these compounds (Marrs *et al*., 1995). Further evidence for ligandin activity in anthocyanin transport is the ability of the petunia An9 protein to complement the *Bz2* mutation, despite the inability of An9 to produce detectable [$^{35}$S]anthocyanin conjugates. Together, these results suggest that only ligandin activity is necessary for anthocyanin transport in these systems (Alfenito *et al*., 1998; Mueller *et al*., 2000). In addition to anthocyanins, GSTs have been shown to bind other flavonoids *in vitro* and be important for their transport (Mueller *et al*., 2000; Winefield *et al*., 2006).

A proteomics approach, combining affinity chromatography and mass spectrometry (MS)/Edman sequencing was used to clone GSTs from pigmented *Vitis vinifera* cell cultures as putative anthocyanin transport proteins. Characterization of these proteins in *V. vinifera* will enable a better understanding of how transport influences accumulation of anthocyanins in the vacuole. This may assist in the augmentation of anthocyanin accumulation in plant cell cultures, as previous methods including precursor feeding, end-product removal, various biotic and abiotic treatments, and overexpression of transcription factors and enzymes in the anthocyanin biosynthetic pathway have been largely unsuccessful (Grotewold *et al*., 1998; Zhang *et al*., 2002*a*; Grotewald, 2006). This may also facilitate more widespread manipulation of the plant metabolome, enhancing accumulation of desirable compounds including glutathione-bound aromatic compounds (Winefield *et al*., 2006) and other flavonoids.

## Materials and methods

### *Plant cell culture and anthocyanin induction*

*Vitis vinifera* cv. Gamay Fréaux var. Teinturier suspension cell line FU-01 was maintained under conditions previously described

(Zhang *et al*., 2002*b*). The FU-01 cell line, selected for anthocyanin production in the dark, was originally developed and characterized by Cormier *et al*. (1994).

After 4 d of incubation, cultures were induced by constant irradiation with cool white light (96.8 ± 2.2 μmol $s^{-1}$ $m^{-2}$), 10 μM jasmonic acid, and 20 g $l^{-1}$ sucrose. Control cultures received equal volumes of vehicle solutions in place of jasmonic acid and sucrose.

### *Growth and anthocyanin content analysis*

Growth of cultures was measured by determining dry cell weight (DCW). Briefly, fresh cells were vacuum-filtered, washed with milliQ water, and transferred to pre-weighed aluminium foil squares. Fresh cell weight (FCW) was recorded and the foil parcels were dried at 90 °C for at least 48 h prior to DCW measurement.

Anthocyanins were extracted quantitatively from fresh, filter-dried suspension cells with 20 vols of acetic acid:$H_2O$ (1:1, v/v) for 1 h at room temperature. Extracts were clarified by centrifugation at 12 000 *g* for 5 min and the supernatant diluted 1:4 (v/v) in 40 mM $Na_2HPO_4$:80 mM citrate buffer, pH 3.0. The absorbance at 535 nm was measured on a Shimadzu UV mini 1240 UV-Vis spectrophotometer, and anthocyanins were quantified in terms of colour value (CV), as described previously (Zhang *et al*., 2002*b*). Following determination of the FCW to DCW ratio, CV/g-DCW was calculated.

### *Analysis of total soluble solids*

*Vitis vinifera* cv. Shiraz berries from the Coombe Vineyard, Waite campus, Adelaide University, were analysed by a hand refractometer (Rochert, Buffalo, NY, USA) by crushing individual berries onto the optical path. For each condition, six berries were quantified (°Brix), and the results are presented as the mean ±SD.

### *Glutathione affinity chromatography*

Glutathione affinity chromatography was performed on the 60% ammonium sulphate precipitate of whole-cell protein extracted from day 5 cultures with GS-Trap columns (GE Healthcare) according to the manufacturer's instructions. Specifically, the flow rate was set to 0.3 ml $min^{-1}$ and recirculated overnight at 4 °C using a P-1 peristaltic pump (GE Healthcare). Washes and elution (10 mM glutathione) were performed at 1 ml $min^{-1}$ using the ÄKTAPrime™ Plus LC system (GE Healthcare) and monitored with the online UV/conductivity recorder at 280 nm.

### *GST assay*

The method of Habig *et al*. (1974) was used to assay for GST activity on protein extracts, with the following modifications. The spectrophotometric assay was performed over 2 min in 1 ml quartz cuvettes with a final concentration of 3 mM GSH and the model substrate 1 mM 1-chloro-2,4-dinitrobenzene (CDNB; ε = 9.2 $mM^{-1}$ $cm^{-1}$) in 100 mM phosphate buffer, pH 7.4.

### *Two-dimensional polyacrylamide gel electrophoresis*

Protein samples were purified with the 2D-Plus One Cleanup Kit (GE Healthcare) and the pellet solubilized in 7 M urea, 2 M thiourea, 0.5% IPG buffer, pI 3–10 (GE Healthcare), 4% CHAPS, and 0.4% dithiothreitol (DTT). Thirteen centimeter IPG strips (pI3-10NL, GE Healthcare) were rehydrated in the above protein solution and focused for 48 kVh. Proteins were separated on a 10% polyacrylamide gel and subsequently stained with SYPRO Ruby (GE Healthcare) or by silver staining (Sambrook and Maniatis, 1989). The BioRad precision plus dual colour protein marker (BioRad) was run as the molecular weight standard.

### *Protein identification*

*MS fingerprinting*: Protein spots were excised from SYPRO Ruby-stained gels with a sterile scalpel and placed into separate sterile Eppendorf tubes. Samples were sent to the Bioanalytical Mass Spectrometry Facility, University of New South Wales, Sydney, Australia, for in-gel trypsin digestion and nano-LC MS/MS analysis as described by Zhao *et al*. (2004).

*Edman sequencing*: Two-dimensional gel electrophoresis was performed as previously mentioned, with the gels pre-run in 1 mM thioglycolic acid. The gel was subsequently equilibrated in 10 mM CAPS buffer and transferred by semi-dry apparatus to nitrocellulose membranes. Membranes were Coomassie stained, dried, and spots excised for Edman sequencing. Automated Edman degradation was performed using a Beckman sequencer (model LF 3400) with online analysis on a Beckman system Gold HPLC (Beckman Coultier).

### *Sequence analysis*

DNA sequencing was performed by the dideoxynucleotide chain termination reaction (BigDye Terminator version 3.1). Once verified, coding sequences were translated using the ExPaSy interface (http://au.expasy.org/tools/dna.html) and protein descriptives obtained with the PeptideMass tool (http://au.expasy.org/tools/peptide-mass.html) using default settings, enabling prediction of the molecular weight and pI of unmodified proteins (Wilkins *et al*., 1997; Gasteiger *et al*., 2005). Pairwise alignments were performed with BestFit alignment using default settings via the BioManager on ANGIS (http://www.angis.org.au). Multiple sequence alignments were produced with a Web-based version of ClustalW (http://align.genome.jp, Thompson *et al*., 1994) using default settings (Matrix = BLOSUM; GAPOPEN = 10, GAPEXT = 0.05). The phylogenetic tree was calculated using the Neighbor-Joining method with PHYLIP via the same website and visualized with Treeview version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

### *Nucleic acid extraction and reverse transcription*

Genomic DNA was extracted from 0.5 g-FCW of FU-01 suspension cells, harvested after 7 d of culture with a Nucleospin® Plant II DNA extraction kit (Macherey-Nagel). Total RNA was extracted from frozen, ground cell material using Concert RNA Reagent (Invitrogen, Carslbad, CA, USA) according to the manufacturer's instructions, and the resultant pellet was resuspended in 30 ml of diethylpyrocarbonate (DEPC)-treated water. RNA samples were quantified using a Genequant Pro nucleic acid spectrophotometer (GE Healthcare). Samples were DNase treated with RQ1 RNase-free DNase I (1 U; Promega, Madison, WI, USA) in 50 ml total volume with RQ1 buffer. RNA was then re-extracted using Trizol reagent (Gibco-BRL, Carlsbad, CA, USA) and resuspended in 10 ml of DEPC-treated water. Sample quality was verified by agarose gel electrophoresis. Reverse transcription was carried out with 500 ng of DNase-treated RNA using 100 U of Superscript III reverse transcriptase (Invitrogen) and oligo$(dT)_{20}$ as per the manufacturer's instructions. DTT was omitted from the buffer due to its impact on SYBR-green 1-based quantitative real-time PCR (qPCR) (Deprez *et al*., 2002).

### *Amplification of full-length GST genes*

Primers were designed to amplify the open reading frame (ORF) of *V. vinifera* GST genes and incorporate restriction sites with Platinum *Pfx* DNA polymerase (Supplementary Table S1 available at *JXB* online) (Applied Biosystems) from both cDNA and genomic DNA. These were A-tailed (Sambrook and Maniatis, 1989) and ligated into pGEM-T Easy (Promega), with three clones sequenced

to check for correct amplification. These were directionally cloned into pQE9 (N-terminal HIS fusion vector, Qiagen) and pJD288 (35S expression vector, Luehrsen *et al*., 1992).

*VvGST3* was cloned by differential display using the Delta Differential Display Kit (Clontech) on *Botrytis cinerea*-infected and mock-infected grapevine plantlet leaves as per Bézier *et al*. (2002). A 380 bp fragment was identified possessing a plant GST gene-like C-terminus following BLAST database searches. Based on this 3′ sequence, two gene-specific primers, GST3R112 (5′-ACCATGCC-TTCACGTGTTTC-3′) and GST3R174 (5′-CAAGAGGGGCCAT-CTAGAGC-3′), were used in two successive RACE (rapid amplification of cDNA ends)-PCR amplifications, as described by the manufacturer (SMART-RACE cDNA Amplification Kit, Clontech), to amplify the 5' region of this transcript. The resulting 800 bp fragment was cloned in pGEM-T II vector (Promega) and three clones were sequenced.

### *Maize kernel bombardment*

Kernels were bombarded according to the method of Alfenito *et al*. (1998).

### *Expression analysis by qPCR*

Primers for phenylalanine ammonia-lyase (*VvPAL*, X75967), chalcone synthase (*VvCHS*, X575969), and UDP-glucose:flavonoid glucosyltransferase (*VvUFGT*, X75968) were each designed against full-length *V. vinifera* cDNA clones (Sparvoli *et al*., 1994). Primers are shown in Supplementary Table S2 at *JXB* online. Dihydroflavonol 4-reductase (*VvDFR*, X75964) and β-tubulin 1 (*VvTUB*, TC4564) primer sequences were provided by personal communication from Dr John Harvey (CSIRO Plant Industry, Adelaide), while primers for the grape berry housekeeping gene *VvUbiquitin1* (BN000705) were previously described by Bogs *et al*. (2006). Samples were performed in duplicate. Amplification, detection, and relative quantification of gene expression using the ΔΔ-Ct equation developed by Pfaffl (2001) was performed using SYBR-green as described previously (Conn *et al*., 2008).

### *Accession numbers*

Sequence data from this article have been deposited with the GenBank/EMBL databases under accession numbers EF088687 (VvGST2), EF469244 (VvGST3), AY971515 (VvGST4), and EF140721 (VvGST5).

## Results

### *Purification of GSTs from grape cell cultures*

GST activity of anthocyanin-accumulating *V. vinifera* L. Gamay Freaux suspension cells fluctuated over time, reflecting the combined activities of individual GSTs during the culture period (Fig. 1a). Both GST activity and anthocyanin accumulation were enhanced by treatment with sucrose, jasmonic acid, and light (Figs. 1a and 5a, respectively). Glutathione-binding proteins were purified using glutathione affinity chromatography on protein from 5-day-old cells, corresponding to high GST activity (Fig. 1a) and the highest rate of anthocyanin accumulation in both treated and control cultures (data not shown). Control cultures, rather than treated cultures, were used for purification of GSTs in order to identify those proteins necessary and sufficient for anthocyanin transport. Multiple

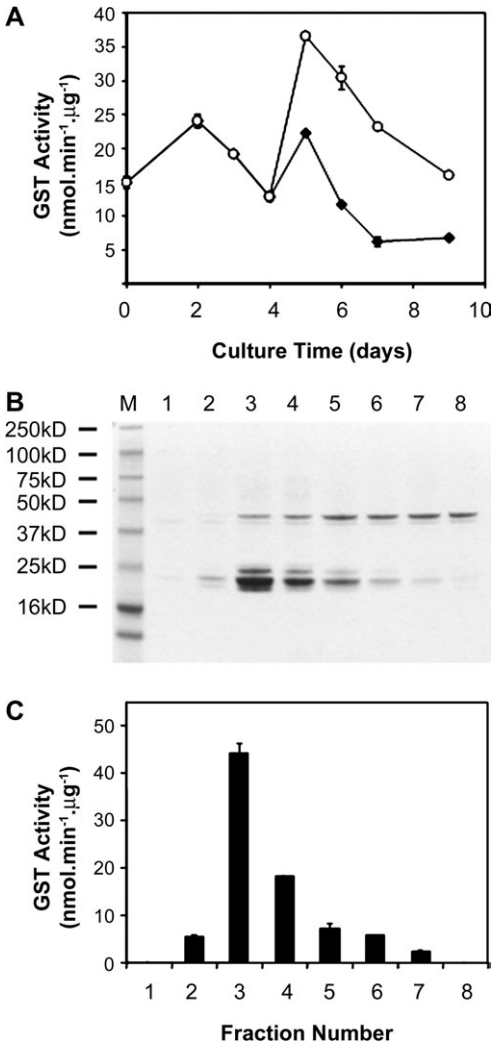


**Fig. 1.** Purification of glutathione-binding proteins from pigmented *V. vinifera* cell suspension cultures. (A) *Vitis vinifera* FU-01 cells were cultured in GC-2 medium (filled circles) for 4 d, then elicited with 10 μM jasmonic acid, 20 g $l^{-1}$ sucrose (or an equal volume of vehicle control), and constant white light irradiation (open circles, 96.8±2.2 μmol $s^{-1}$ $m^{-2}$). Cultures were incubated at 27±1 °C on a reciprocating shaker at 100 strokes $min^{-1}$, in 500 ml Erlenmeyer flasks containing 100 ml of B5 medium (Gamborg *et al*., 1968) supplemented with 30 g $l^{-1}$ sucrose, 250 mg $l^{-1}$ casein hydrolysate, 0.1 mg $l^{-1}$ α-naphthaleneacetic acid, and 0.2 mg $l^{-1}$ kinetin. GST activity, presented as the mean ±SD of three biological replicates performed in triplicate, was determined for the 60% ammonium sulphate precipitate ($n$=9). (B) Sixty percent ammonium sulphate precipitate from day 5, non-elicited FU-01 line subjected to GST affinity chromatography. One-dimensional gel electropherogram of fractions (0.5 ml) from the GSTrap column. (C) Corresponding GST activity on the same fractions using CDNB as the model substrate.

proteins were identified in the fractions displaying GST activity by one-dimensional gel electrophoresis (1D-GE; Fig. 1b). The GST activity of the fractions was proportional to the prevalence of proteins with a molecular weight of 21–26 kDa (Fig. 1b, c). The 45 kDa band, present in most fractions, may represent another glutathione-binding protein, with no apparent correlation with GST activity. GSTs

were estimated to comprise >95% of protein in the bound fractions from the 1D-GE (equivalent to 2.3% of total protein from *V. vinifera* cell cultures).

### *Two-dimensional gel electrophoresis and peptide mass fingerprinting*

Fractions from the glutathione affinity chromatography with GST activity were pooled and separated by 2D-GE (Fig. 2). Proteins were visualized using the MS-compatible SYPRO Ruby stain, following verification that over-exposed silver staining (data not shown) did not indicate the presence of otherwise undetected low abundance proteins.

Proteins were digested in-gel with trypsin, and a mass spectrum of the resulting peptides (a peptide mass fingerprint) was acquired with a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer as per Zhao *et al.* (2004). The list of apparent peptide masses was then aligned with predicted tryptic digests of known proteins by MASCOT and BLASTp (Altschul *et al.*, 1990). A combination of low abundance and/or poor ionization did not enable the analysis of individual peptides by MS/MS.

This alignment enabled grouping of protein spots based on identical peptide fingerprints, with the variation in pI within each group a result of carbamylation of lysine residues diagnosed by the MS profile, a common artefact during the 2D separation procedures (McCarthy *et al.*, 2003). Two groups (α, ε) required Edman sequencing to identify the N-terminus for stronger inference of the candidate expressed sequence tag (EST). The N-terminal sequence for group α did not enable robust degenerate PCR primer design, thus the gene designated as *VvGST3* was obtained by 5′ RACE-PCR from its 3′ EST which had been identified by a single matching peptide fragment. The cloned gene matched the N-terminal sequence obtained by Edman degradation for this protein.

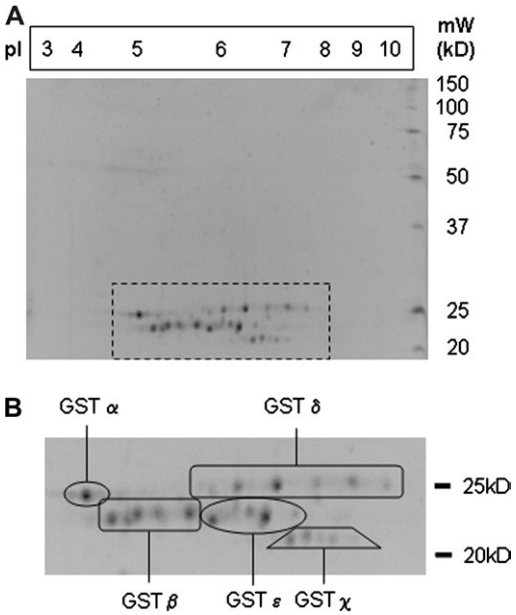


**Fig. 2.** (A) Two-dimensional gel electropherogram of glutathione affinity chromatography fractions possessing GST activity. (B) Enlarged image of the boxed region showing grouping based on mass spectrometry fingerprints (refer to Table 1). IEF strip pI3-10NL was rehydrated in the protein precipitate from GST active fractions of the GSTrap column. This was focused for 48 kVh and then separated on a 10% polyacrylamide gel and stained with SYPRO Ruby.

Once a contig was generated within The Institute of Genomic Research (TIGR) grape database, all GSTs were amplified from pigmented *V. vinifera* suspension cell cDNA. *VvGST1*, *VvGST2*, *VvGST3*, and *VvGST4* all produced single PCR products of the predicted size, while *Q84N22* yielded two PCR products. While the longer product possessed numerous stop codons in all frames, the shorter PCR product possessed 99% identity with the published *Q84N22* sequence. Of note is a frameshift mutation common in all clones sequenced (AAA, instead of the published AAAA), altering all amino acids downstream of Leu45 and the predicted protein molecular weight for the Q84N22 EST from 15.7 kDa to 25.5 kDa. This agreed with the molecular weight of proteins purified from the cell culture (Figs 1, 2), and therefore this coding sequence and protein are hereafter referred to as VvGST5.

Amplification of GST gene sequences from genomic DNA revealed the presence of a single intron in both *VvGST1* and *VVGST5*, two introns in *VvGST2* and *VvGST3*, while *VvGST4* did not contain an intron (Fig. 3a). Alignments with other plant GST proteins indicate VvGST4 to be a type I GST (Fig. 3b) and support the classification of the other GSTs based on intron/exon structure alone.

### *Functional expression of genes encoding VvGSTs*

Recombinant expression of these sequences was performed to confirm GST activity. Each cDNA clone was expressed in *Escherichia coli* with an N-terminal 6×HIS fusion to facilitate purification and western blotting. The fusion products were of the predicted size, and GST activity was confirmed in crude extracts from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *E. coli* for all genes (Fig. 4) and also following partial enrichment by Ni-NTA chromatography, showing activity similar to that of other recombinant GSTs cloned from poppy and petunia (data not shown; Alfenito *et al.*, 1998; Yu and Facchini, 2000).

### *Transcription of GSTs and anthocyanin biosynthetic genes in cultured cells following induction with sucrose, jasmonic acid, and light irradiation*

Treatment of *V. vinifera* cell suspension cultures with continuous white-light irradiation, sucrose, and jasmonic acid from day 4 increased the anthocyanin content

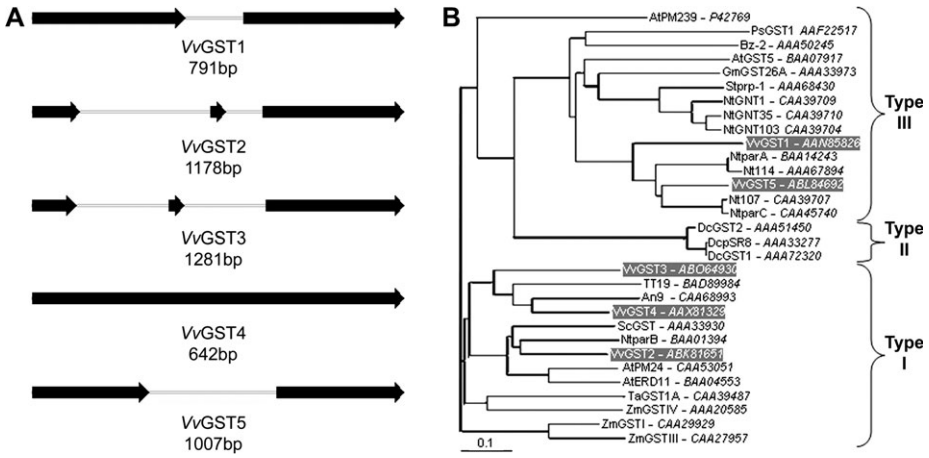


**Fig. 3.** (A) Genomic structure of *V. vinifera* GSTs cloned in this study. Exons are indicated by black arrows, and introns are indicated by double lines. (B) Phylogenetic tree of GSTs in the plant kingdom, showing classification into type I, II, and III GSTs as per Droog (1997). Sequences aligned are listed on the right-hand side of the tree and were obtained by retrieval of protein sequences from GenBank. Multiple sequence alignments were created by ClustalW (Thompson *et al*., 1994) and the phylogenetic tree was generated using PHYLIP under default settings and viewed with Treeview version 1.6.6. Origins of the proteins are indicated by a two-letter prefix to the protein name: At, *Arabidopsis*, Dc, carnation; Nt, tobacco; Sc, cucumber; St, potato; Ta, wheat; Vv, grape (highlighted); Zm, maize. An9, TT19, and Bz2 are shown without prefixes for consistency.

(Fig. 5a) compared with control cultures at day 8. Jasmonic acid was the most effective single treatment, eliciting a 3.1-fold increase, while induction with all three treatments in combination yielded the maximum increase in anthocyanin content, ~6-fold that in control cultures. Over the 96 h immediately following induction, distinct temporal patterns of transcript abundance for key anthocyanin biosynthetic genes (Fig. 5b) and putative GST genes (Fig. 5c) were observed in induced cultures, indicating differential regulation. Transcription of the first committed precursor pathway gene *PAL* increased to a maximum 12 h after induction, whereas transcripts for committed steps to flavonoids (*CHS*), leucocyanidins (*DFR*), and anthocyanins (*UFGT*) were co-ordinately up-regulated, with maxima at 24 h post-induction (Fig. 5b). The degree of up-regulation was comparable for each gene, within the range of 7- to 12-fold that in control cultures.

Under the induction conditions used here to enhance anthocyanin biosynthesis, all putative grape GST genes, with the exception of *VvGST5*, were up-regulated relative to control cultures (Fig. 5c). The greatest degree of up-regulation (12-fold) was observed for *VvGST1*, exhibiting a temporal pattern similar to *PAL*. Up-regulation of *VvGST3* transcription also followed this pattern, although it was not as strongly induced (5.5-fold) over controls. The pattern of up-regulation for *VvGST4* was similar to that of *CHS*, *DFR*, and *UFGT*, while *VvGST2* exhibited delayed and sustained up-regulation of transcription (Fig. 5c).

### *Transcription of GST genes in grape berry skins at veráison*

Bunches of *V. vinifera* L. cv. Shiraz berries were obtained from the same vine at veráison from predominantly green (<10% lightly pigmented berries, which were not chosen

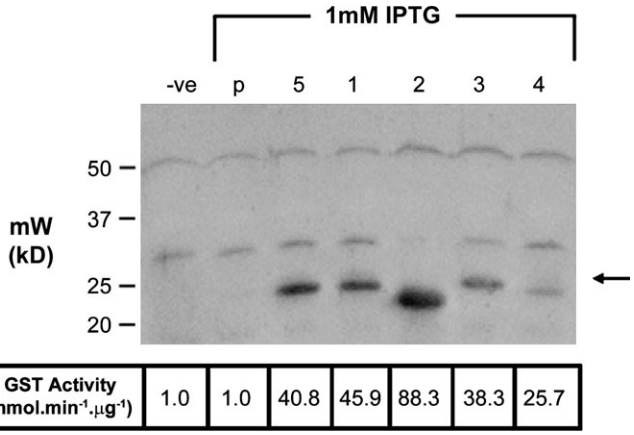


**Fig. 4.** Western blot and GST activity performed on crude soluble protein extract from *E. coli* carrying vectors as indicated. –ve, *E. coli* carrying pQE9:*VvGST5* without IPTG induction; p, *E. coli* carrying the pQE9 backbone only; GST coding sequences in pQE9 5, VvGST5; 1, VvGST1; 2, VvGST2; 3, VvGST3; 4, VvGST4. The arrow indicates the 6×HIS fusion product. M15:pREP4 *E. coli* were transformed with *VvGST* cDNAs in pQE9, N-terminal HIS fusion vector (Invitrogen) and grown at 37 °C. Induction of HIS fusion proteins was achieved with 1 mM IPTG for 18 h, with soluble protein extracted according to Sambrook and Maniatis (1989). Western blotting was performed using an anti-HIS detection kit (Macherey-Nagel).

for extraction) and fully coloured bunches. The measure of total soluble solids for the green and red Shiraz berries was calculated as 6.4±0.3 °B and 14.8±1.4 °B, respectively. While no anthocyanin was detected by high-performance liquid chromatography (HPLC) from green berries, the red berry skins had an anthocyanin content of 1.4±0.05 mg g-FCW$^{-1}$. These measurements justified the attribution of them as pre- and post-veráison stage berries (Kennedy *et al*., 2000).

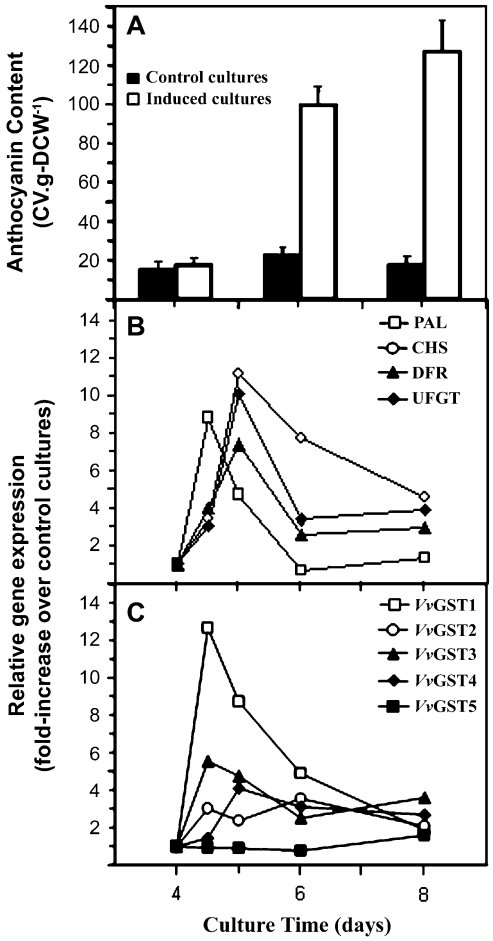


**Fig. 5.** Anthocyanin accumulation and relative mRNA transcript levels for key anthocyanin biosynthetic genes in cultures following induction after 4 d with sucrose, jasmonic acid, and continuous light irradiation. (A) Anthocyanin content in *V. vinifera* cell suspensions following induction, compared with control cultures. Data are presented as the mean ±SD of triplicate analyses. The fold increase in steady-state transcript was calculated using the ΔΔ-Ct equation (Pfaffl, 2001), with β-tubulin as internal control, for (B) phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP-glucose:flavonoid glucosyltransferase (UFGT), and (C) glutathione *S*-transferases 1–5 (VvGST1–5). All data are expressed relative to untreated cultures at each time point and are presented as the mean of duplicate analyses.

Expression of *VvGST4*, *VvGST3*, and *VvGST2* was found to be greater—60-, 2-, and 1.4-fold, respectively—in the post-veraison berry skins, concurrent with the accumulation of anthocyanin in this tissue (Fig. 6). In contrast, *VvGST1* was the most strongly induced (~13-fold) when suspension cell cultures were elicited to accumulate more anthocyanins. In grape berry skins, *VvGST1* and *VvGST5* expression was unaffected. Taken together, qPCR transcription analyses suggest a strong correlation of GST up-regulation with anthocyanin accumulation. Differences in the transcription profile may arise from interspecies variation, or a differential response to specific stimuli.

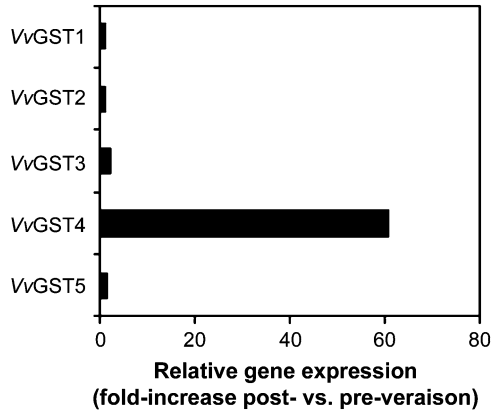


**Fig. 6.** Relative mRNA transcript levels for GSTs in post-veraison *V. vinifera* L. cv. Shiraz berry skins compared with pre-veraison. Fold increase in steady-state transcripts calculated using the ΔΔ-Ct equation, with *VvUbiquitin1* as internal control. Data are presented as mean of triplicate analyses.

### *VvGST1 and VvGST4 are involved in anthocyanin accumulation in the vacuole*

A complementation model for anthocyanin transport, based on reversion of the *bz2* phenotype, was utilized to determine the ability of the putative *V. vinifera* L. GSTs to transport cytosolic anthocyanins to the vacuole. Each GST was transiently expressed in *bz2* kernels to identify revertant zones (Fig. 7). These zones can be easily distinguished from the background and necrotic areas resulting from the high pressure bombardment due to the non-delimited anthocyanin deposition, appearing as a 'halo' of red pigmentation (Alfenito *et al*., 1998; Larsen *et al*., 2003). Of the grape GST genes tested, *VvGST1* exhibited the greatest degree of *bz2* complementation (37 zones $mm^{-2}$, Table 2) and an average diameter of the revertant zones approaching that of *Bz2*, at 3.2 and 3.6 cell widths, respectively.

VvGST4 was the only other GST tested to exhibit the complemented phenotype (10 zones $mm^{-2}$) and a greatly reduced average diameter of these zones (2.3 cell widths, Table 2). All other VvGSTs and an unrelated GST from *Arabidopsis thaliana* possessed a background level of necrosis (Fig. 7) due to the bombardment, but no characteristic anthocyanin 'halo'.

### *Conservation of protein residues found among plant GSTs is linked to the rescue of anthocyanin transport*

Bestfit alignments were made between the amino acid sequence of all *V. vinifera* GSTs and those plant GSTs previously demonstrated to complement the *bz2* mutant; An9, TT19, and Bz2 (Table 2; Marrs *et al*., 1995; Alfenito *et al*., 1998; Mueller *et al*., 2000). It was found that VvGST1 possessed the second highest similarity to Bz2 (48.2% similarity), while the most similar sequence, VvGST5 (48.3% similarity), did not complement *bz2* in

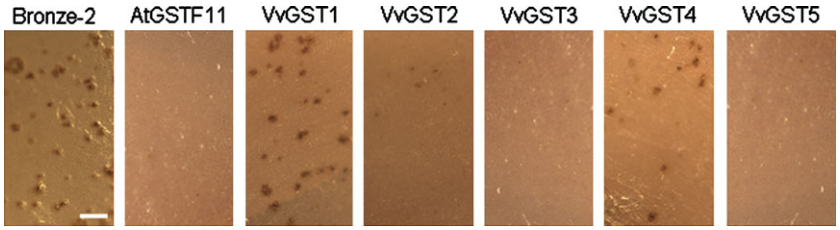


**Fig. 7.** Complementation of anthocyanin transport by *V. vinifera* GSTs in *Bronze-2*-deficient corn kernels. Corn kernels 48 h after bombardment with GST constructs in pJD288 (35S) maize expression vector; 20× magnification. Bar = 300 μm. *Bronze-2*-deficient corn kernels were bombarded with 1 μm diameter gold particles coated with plasmid, and analysed as per Alfenito *et al*. (1998). Images were captured using an Olympus BX50 light microscope equipped with a Canon EOS digital camera.

**Table 1.** *Peptide fingerprint analysis identifying the proteins as GSTs*

Peptide fingerprints of *V. vinifera* glutathione-binding proteins separated by 2D-GE and analysed by MS and Edman sequencing. Alignment of the predicted peptide fingerprint sequences with known ESTs from public databases (GenBank, ExPaSY, and TIGR; see Fig.2 for grouping) to identify the strongest match.

| Group | Strongest match | Amino acid region | Mass fingerprints | Matching grape GST [accession number(s)] |
|---|---|---|---|---|
| a | *Glycine max*, Q9FQE5 | 1–11 | MVVKVYGPDFA[a] | VvGST3 [NP864089, AAG34814, AY156050] |
| | | 205–210 | KVFSSR | |
| b | *Vitis vinifera*, Q84N22 | 41–52 | SPLLLEMNPVHK | VvGST5 [AF501625] |
| | | 106–111 | LYELGR | |
| | | 139–149 | LGEKPYFGGEK | |
| c | *Arabidopsis thaliana*, AF288176 | 17–25 | RVVAALYEK | VvGST2 [TC44916,TC14045, TC25424] |
| | | 65–69 | LFESR | |
| | | 144–151 | VLDVYEAR | |
| d | *Oryza sativa*, AAP54756 | 87–95 | LLPADPYQR | VvGST1 [AY156048] |
| | | 137–143 | MLEGELK | |
| e | *Arabidopsis thaliana*, AC013430 | 1–7 | MVMKVYG[a] | VvGST4 |
| | | 143–150 | VFDVYEQR | [TC39256] |

[a] Sequence obtained by Edman sequencing.

**Table 2.** *Quantitative complementation of* Bz2 *knockout corn seeds by* V. vinifera *GSTs*

Counts of complementation zones seen per 1 $mm^2$ field. $n$=3 experiments with triplicate seeds, with 10 fields counted per seed. Counts are presented as mean ±SD. Protein sequence similarity between anthocyanin-transporting GSTs and sequences utilized in the complementation assay (BestFit).

| Construct | Sectors per $mm^2$ field | Complementation zones (% of each category) | | | Average spot diameter (cells) | Peptide similarity to anthocyanin-transporting GSTs | | |
|---|---|---|---|---|---|---|---|---|
| | | 1- to 2-cell diameter | 3- to 4-cell diameter | >5-cell diameter | | Maize (Bronze-2) | *Arabidopsis* (TT19) | Petunia (An9) |
| Control plasmid | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A |
| Bronze-2 | 15.6±0.9 | 22.7% | 60.8% | 16.5% | 3.6±0.18 | 100% | 35.5% | 37.2% |
| AtH36860 | 0 | 0 | 0 | 0 | 0 | 37.1% | 76.2% | 64.0% |
| VvGST1 | 14.3±0.6 | 31.9% | 56.3% | 11.8% | 3.2±0.13 | 48.2% | 36.9% | 34.4% |
| VvGST2 | 0 | 0 | 0 | 0 | 0 | 39.6% | 49.3% | 48.3% |
| VvGST3 | 0 | 0 | 0 | 0 | 0 | 37.8% | 59.2% | 66.0% |
| VvGST4 | 3.9±0.7 | 56.5% | 43.5% | 0 | 2.3±0.20 | 37.7% | 68.4% | 75.8% |
| VvGST5 | 0 | 0 | 0 | 0 | 0 | 48.3% | 37.8% | 41.8% |

N/A, not applicable.

this assay. VvGST4 was most similar to An9 and TT19, 75.8% and 68.4% similarity, respectively. VvGST4 also gave a similar level of complementation in the maize bombardment assay to these GSTs (Alfenito *et al*., 1998), inferring that the protein sequence was influential in governing anthocyanin transport in the *bz2* complementation model. All VvGST sequences were aligned with Bz2, TT19, ZmGSTIII, and An9 to identify conserved residues (Fig. 8). Few key regions were identified as being unique to anthocyanin-transporting GSTs, with other single or

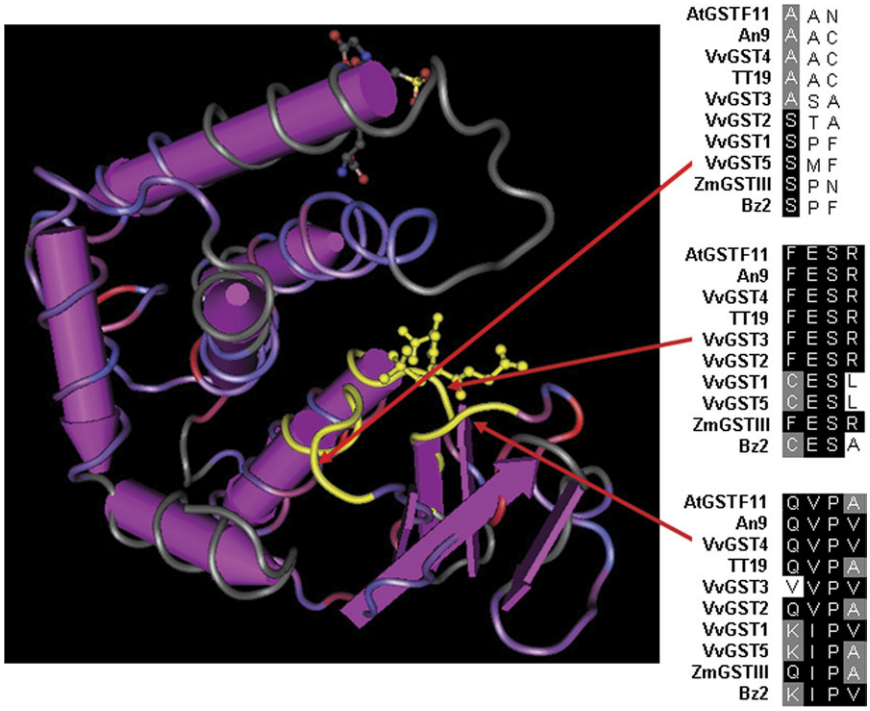


**Fig. 8.** Inference of functional residues in anthocyanin transport by protein modelling. Three-dimensional model of *E. coli* GST chain A, interacting with its substrate (glutathione sulphonic acid). The highlighted regions are within 3 Å of the substrate and expanded to show the respective coding sequences for the aligned GSTs. Alignment of VvGSTs with known anthocyanin-transporting GSTs complementing the *bz2* maize model. Amino acids shaded in black are present in ⩾50% of the proteins at that residue, while alternative conserved residues at that site are shaded in grey, with other unique residues left unshaded.

double amino acid residues differing between GSTs with low and high activity in the *bz2* complementation assay.

These regions were located on a three-dimensional model of two GSTs interacting with non-substrate ligands. The primary crystal structure used for this alignment was the *E. coli* GST chain A, complexed with the ligand glutathione sulphonic acid (Nishida *et al*., 1998) as the sequence was most similar to the VvGSTs, but sequences were also overlaid with the maize ZmGSTI complexed with lactoylglutathione (Neuefeind *et al*., 1997). Three of these homologous regions (ligand-binding sites, L-sites) were located on the outside of the protein between α-helices and within 3 Å, or in proximity for physical interaction with these ligands (Fig. 8).

There is a strong conservation in the core of these regions between VvGST1 and Bz2, while there are residues that differ from other anthocyanin-transporting GSTs. Also, residue differences between the highly similar VvGST5 and VvGST1 sequences fall in two of these regions (Fig. 8).

VvGST4, An9, and, to a lesser extent, ZmGSTIII (all type I GSTs) exhibit a similar response in the complementation assay (Alfenito *et al*., 1998). As well as high overall similarity, these GSTs possess strong homology in the aforementioned mini-regions, but differ from the GST1/Bz2 mini-regions and also for the non-transporting AtGSTF11. This includes high similarity within the glutathione-binding sites (G-sites) and L-sites.

## Discussion

Cloning of plant GSTs to date has predominantly been achieved through large-scale mutagenesis experiments, EST and differential display approaches, or genome sequencing projects such as with *Arabidopsis*, rice, and maize (McGonigle *et al*., 2000; Alonso *et al*., 2003). Affinity chromatography was used to purify two GSTs from oat seedlings, although they were only partially characterized; one GST from *Z. mays* was cloned via screening a cDNA library with antisera against the purified protein, and a single type III GST was cloned by degenerate PCR from opium poppy suspension cells (Dixon *et al*., 1998; Yu and Facchini, 2000; Zachariah *et al*., 2000). This is the first report of a proteomics approach for the purification and cloning of GSTs from any plant tissue. Anthocyanin-producing *V. vinifera* cv. Gamay Freaux cell suspension cultures were used to characterize anthocyanin transport proteins as they produce the same anthocyanin profile as in the berry, and for the ability to manipulate anthocyanin production in culture (Dornenburg and Knorr, 1996; Yeoman and Yeoman, 1996; Verpoorte *et al*., 1999).

The number of GST-like sequences found in different plant species ranges from 25 in soybean to 42 and 48 in maize and *Arabidopsis*, respectively (McGonigle *et al*., 2000; Dixon *et al*., 2002; Wagner *et al*., 2002). Pairoba and Walbot (2003) demonstrated that the 26 kDa Bz2 protein is accumulated only in anthocyanin-accumulating tissues, suggesting the presence of functional anthocyanin-specific GST protein in pigmented grape cells. Therefore, while this study cannot be considered exhaustive with regards to characterization of all the GSTs from *V. vinifera*, it is a comprehensive study of functional anthocyanin transport. Of the five GST sequences cloned in this study from pigmented suspension cells, there were three type I GSTs (VvGST2, VvGST3, and VvGST4) and two type III GSTs (VvGST1 and VVGST5) based on intron structure and protein sequence alignments.

Anthocyanin accumulation, including post-biosynthetic steps such as transport into the vacuole, has been shown in various systems to be regulated at the transcriptional level (Matsui *et al*., 2004; Hirai *et al*., 2005; Castellarin *et al*., 2006). It was hypothesized that if GSTs were involved in anthocyanin transport in *V. vinifera*, they would be present and transcriptionally up-regulated during anthocyanin synthesis. Using both *V. vinifera* suspension cells and grape berries, qPCR was employed to determine which of the five GSTs identified could be responsible for transporting anthocyanins in grape cells, through observation of co-regulation with anthocyanin biosynthetic genes and correlation to anthocyanin accumulation.

Biotic and abiotic elicitation is a common methodology for probing of metabolic pathways in *in vitro* systems, and a combination of jasmonic acid and light irradiation has been demonstrated to enhance anthocyanin biosynthesis in *V. vinifera* suspension cultures (Zhang *et al*., 2002*b*). High-sucrose stress, known to influence anthocyanin synthesis (Cormier *et al*., 1990; Solfanelli *et al*., 2006), in combination with jasmonic acid and light after 4 d of culture, enhanced anthocyanin accumulation >6-fold and enabled robust transcriptional profiles to be obtained for key anthocyanin biosynthetic genes. Differential regulation of *PAL* compared with later pathway genes was not unexpected. The most responsive GST gene to these conditions, *VvGST1*, also displayed a transcriptional profile similar to that of *PAL*, while the profile for *VvGST4* was more similar to that of the late anthocyanin pathway genes, *CHS*, *DFR*, and *UFGT*, indicative of co-regulation. In orange fruit, the expression of a putative anthocyanin-transporting GST also correlated with the expression of the *PAL*, *CHS*, *DFR*, and *UFGT* genes under cold stress (Lo Piero *et al*., 2005, 2006).

Both C1 (Myb DNA-binding factor) and R (bHLH domain of Myc) transcription factor-binding elements reside in committed maize anthocyanin biosynthetic genes (Dooner *et al*., 1991). These elements have also been found in a 262 bp region of the *Bz2* promoter, sufficient to confer co-ordinate regulation of this gene with those of the biosynthetic pathway (Bodeau and Walbot, 1992, 1996). Only *VvGST1* possesses both of the the C1/R-binding motifs in its 5′-untranslated region. These elements may lead to the altered pattern of transcription seen in suspension cells versus grape berries.

There was no increase in *VvGST5* in suspension cells, thus it was unlikely to be involved in anthocyanin transport under these conditions. Results with post-veráison berries provided further evidence that *VvGST5* was not involved in anthocyanin transport, while the strongest up-regulation was for *VvGST4*, inferring a key role in anthocyanin transport in shiraz grapes. A similar pattern of induction was noted for numerous genes in Shiraz berries by Terrier *et al*. (2005), including a partial EST with high similarity to a GST gene (now known to correspond to *VvGST4*, cloned in this study). Interestingly, *VvGST3* was the only other candidate that exhibited up-regulation in grape berries post-veráison. From the combined data, it appeared that *VvGST1*, *VvGST3*, and *VvGST4* may be involved in anthocyanin transport, but in response to different stimuli—stress and grape berry ripening, respectively.

Complementation of anthocyanin transport in *Bz2*-deficient maize kernels was considered appropriate for inference of anthocyanin transport for VvGST genes as cyanidin 3-glucoside and its acylated derivatives are common anthocyanins in both plants (Cormier and Do, 1993). As in maize, two GST genes—*VvGST1* and *VvGST4*—yielded positive results with this assay, although with different efficiencies.

Sequence analysis for all anthocyanin-transporting GSTs able to complement *bz2* gave no consensus of substrate-binding residues (H-sites), supporting the findings of other researchers (Alfenito *et al*., 1998; Kitamura *et al*., 2004). However, both G-sites and L-sites on the exterior of the protein, thought to be prime candidate regions for binding non-substrate ligands, were shown to be conserved within the VvGST1/Bz2 and VvGST4/An9/ZmGSTIII groups, respectively. These regions were a source of dissimilarity between the otherwise similar VvGST1 and VvGST5, and may contribute to the lack of activity observed for VvGST5 in the maize complementation assay. All these critical binding sites were within 3 Å of the substrate. While ligandin-binding sites have been suggested to reside distinct from the active site (McTigue *et al*., 1995; Ji *et al*., 1996; Oakley *et al*., 1999), competitive activity has been demonstrated between model substrates (CDNB) and ligandins (anthocyanins, auxins), implicating the same binding site, i.e. the L-site (Mueller *et al*., 2000; Smith *et al*., 2003; Lo Piero *et al*., 2006).

VvGST1 possessed the second highest similarity to Bz2 behind the non-complementing VvGST5, and suggests the involvement of distinct residues in achieving anthocyanin

transport. Interestingly, the similarity to Bz2 was proposed as the reason for the slightly higher complementation phenotype of ZmGSTIII as opposed to An9 (Alfenito *et al*., 1998). Certainly, the modification of individual flavonoids, by addition or translocation of a glucoside residue in cyanidin or luteolin, leads to a greatly reduced ability to bind An9 (Mueller *et al*., 2000).

Despite the dissimilarity of the dicot and monocot GST sequences, the strongest complementing GST in the dicot grape (VvGST1) is more similar to the monocot maize GST (Bz2) than any of the other transporting GSTs. The other *V. vinifera* GST capable of transport, VvGST4, possessed the highest similarity to the petunia An9 and yielded a similar result in the *bz2* complementation assay (Alfenito *et al*., 1998). Maize and petunia have different dominant anthocyanin species, cyanidin and petunidin/cyanidin, respectively, with different GSTs required for transport being defined by unique consensus sequences inferred in this study. This may reflect the independent evolution of GSTs with regard to the anthocyanin profile within each species, with the prevalence of five anthocyanin compounds in grape requiring a second GST ligandin. In an evolutionary sense, plant GSTs belong to the most ancient class of GSTs, also present in vertebrates, *Drosophila*, and *Methylobacterium* (Mannervik and Danielson, 1988; Pemble and Taylor, 1992). This suggests that they are a representative of a progenitor GST arising before the divergence of prokaryotes and eukaryotes, maximizing the potential for independent gene duplication/sequence divergence to facilitate catalytic adaptation (Pemble and Taylor, 1992).

As is the case for many secondary metabolite biosynthetic proteins, there is an apparent redundancy with the anthocyanin-transporting GSTs in *V. vinifera*. This redundancy is seen with numerous functional copies of biosynthetic enzymes, including PAL (Ozeki *et al*., 1990), CHS (Reif *et al*., 1985; Durbin *et al*., 2000), and CHI (van Tunen *et al*., 1988); but also the putative anthocyanin transport pumps—ZmMRP3 and ZmMRP4 (Goodman *et al*., 2004). While maize also boasts two GSTs with transport activity, ZmGSTIII and Bz2, a knockout of the latter abrogates anthocyanin vacuolar deposition (Alfenito *et al*., 1998). This demonstrates a dependency on *Bz2*, yet does not eliminate the possibility of (i) *ZmGSTIII* transcriptional repression in these tissues, or (ii) potential heterodimerization with Bz2 for a functional ligandin. Furthermore, co-cultivation with abscisic acid induces vacuolar deposition of anthocyanins in *bz2* maize kernels, supporting a partial redundancy by the auxin-inducible ZmGSTIII for Bz2, despite its reduced transport capacity (Walbot *et al*., 1994; Alfenito *et al*., 1998). Analysis of gene expression for ZmGSTIII in abscisic acid-treated *bz2* kernels, or creation of ZmGSTIII knockout maize would be necessary to resolve this. Either of these models may also apply for VvGST1 and VvGST4 in *V. vinifera*.

Results presented in this study demonstrate the strong correlation between GST proteins, abundance of transcripts encoding them, and anthocyanin accumulation in *V. vinifera*. Specifically, *VvGST1* and *VvGST4* are the most responsive GSTs under synthetic (elicitation) and native (veráison) conditions increasing anthocyanin accumulation, respectively. In support of this, both *VvGST1* and *VvGST4* possess a capacity to complement for the loss of Bz2 in a maize anthocyanin transport complementation assay. Furthermore, the sequence conservation of specific residues in the GSTs contributes to their anthocyanin-transporting phenotype.

Understanding critical regions in the interaction, GSTs can be manipulated to alter or enhance accumulation of desired compounds, i.e. valuable flavonoids. Dixon *et al*. (2005) advocate the heterodimerization/co-expression, chimera formation, and random mutagenesis/recombination of maize GSTs for enhanced herbicide detoxification. Furthermore, overexpression of certain GSTs may enhance the accumulation of endogenous nutraceuticals, or GST repression may lead to enhanced accumulation of pytochelatins (heavy metal-binding proteins, reviewed in Dixon *et al*., 2005). These two opposing effects could result in a food source with greater nutrition in poor quality soils.

## Supplementary data

Supplementary data for this manuscript detailing primers used for directional cloning of all GSTs and qPCR primers used can be accessed at *JXB* online.

## Acknowledgements

The authors would like to thank Professor Virginia Walbot for supplying *bronze-2* corn kernels, Drs Mark Alfenito and Dean Goodman for valuable assistance in complementation analysis, and Professor Antony Bacic and Dr Shaio-Lim Mau at the School of Botany, University of Melbourne for Edman sequencing. This work was supported by Flinders University and the Cooperative Research Centre for Bioproducts.

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