Functional analysis of genes involved in anthocyanin accumulation in *Arabidopsis thaliana*

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ABBREVIATION

A. thaliana	*Arabidopsis thaiana*
3AT	3-amino-1,2,4-triazole
AD	Activation domain
bHLH	basic helix-loop-helix
bp	Base pair
°C	degree Celsius
CaMV	Cauliflower mosaic virus
cDNA	Complementary Deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CoA	Coenzyme A
Col	Columbia Ecotype
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
(μ) g	(micro)gram
GH	Glycine-Histidine
GM	Germination medium
GSH	Glutathione
GST	Glutathione *S*-transferase
h	hour(s)
His	Histidine
kDa	Kilodalton
Leu	Leucine
min	minute(s)
ml	milliliter
mRNA	Messenger Ribonucleic acid
Myb	Myb-type transcription factor

Myc	bHLH-type transcription factor
P. frutescens	*Perilla frutesces*
PAP1	Production of Anthocyanin Pigment 1
pap1-D	Production of Anthocyanin Pigment 1-Dominant
PAs	Proanthocyanidins
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription- Polymerase chain reaction
s	second(s)
SC	Synthetic Complete medium
TAIR	The Arabidopsis Information Resource
T-DNA	Transferred DNA
Trp	Tryptophan
TT	Transparent testa
Ura	Uracil
UV	Ultraviolet
WD	Tryptophan-Aspatate
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YPAD	Yeast Extract - Peptone - Dextrose plus Adenine medium

CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

GENERAL INTRODUCTION

Anthocyanins and its importance

Flavonoids are a large group of phenolic secondary metabolites that are widespread among plants and are involved in many plant functions. Anthocyanins, a flavonoid subclass, are brightly colored pigments produced in flowers and fruits whose main function is to attract pollinators and seed dispersers (Harborne and Williams 2000). Anthocyanins are also protect the tissues from UV damage by absorb ultraviolet (UV) light as well as visible light and have been suggested to serve as UV protectants (Stapleton and Walbot 1994).

Proanthocyanidins are naturally occurring compounds that are widely found in fruits, vegetables, nuts, seeds, flowers and bark of many plants (Dixon *et al*. 2005). It is now generally accepted that proanthocyanidins play a role in protecting plants against insect pests, mammalian herbivores and microbial pathogens (Harborne and Williams 2000).

Interest in anthocyanins and proanthocyanidins increased due to their health-promoting activities. Anthocyanins possess considerable antioxidant properties and are therefore believed to reduce the risk of coronary heart disease and cancer (Wang *et al*. 1997). The low toxicity of these compounds makes them valuable nutraceuticals (Santos-Buelga and Scalbert 2000). Recently, there has been intense interest in the potential of flavonoids to modulate neuronal function and prevent against age-related neurodegeneration (Vauzour *et al*. 2008). There have been reported that anthocyanins also contain anti-inflammatory effect (Harborne and Williams 2000). Moreover,

anthocyanins are widely used as natural food colorants, for example, the cyanidin acylglucosides *Perilla frutescence* (Gong *et al*. 1997, Heci 2001). Proanthocyanidins showed their potent antioxidant capacity and showed protection against free radical-mediated injury and cardiovascular disease (Bogs *et al*. 2005). The antioxidant properties related to their radical scavenging capacity and these properties have been used against heart disease through reducing lipid oxidation (Ricarda Da Silva *et al*. 1991) and also can protect ruminants against pasture bloat (McMahon *et al*. 2000).

Chemistry of anthocyanins

Anthocyanins are water-soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium salts. Anthocyanins can be divided to six major groups: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Figure 1). Their color greatly depends on the number of hydroxyl groups on the B-ring; the larger the number of groups, the bluer the color. *O*-Methylation of anthocyanins has a slight reddening effect. Anthocyanins are most frequently *O*-glycosylated (usually glucosylated) at the C3-position, followed by the C5- position. Glycosylation at C7′, C3′ or C5′ is often found. Glycosylation of anthocyanins results in slight reddening (Tanaka *et al*. 2008).

Proanthocyanidins, or condensed tannin, is a polymeric flavanol that rich in hydrophobic aromatic rings and hydroxyl groups that can interact with biological molecules, particularly proteins, by hydrogen bonds and hydrophobic interactions. This strong interaction of PAs with proteins is probably the basis of their main role in plants and their uses by man (Abrahams *et al*. 2002). Proanthocyanidins are high-molecular-

weight polymers comprised of the monomeric unit flavan-3-ol; (+)catechin and (-) epicatechin. Oxidative condensation occurs between carbon C-4 of the heterocycle and carbons C-6 or C-8 of the attached A and B rings (refer to Figure 1). Basic structure of proanthocyanidins is shown in Figure 2 (Bravo 1998).

Transcriptional regulation of anthocyanin

Flavonoid biosynthesis is the one of the most intensively studied secondary metabolite pathways. The anthocyanin biosynthetic pathway was described in different plants (Holton and Cornish 1995), including *Arabidopsis thaliana* (Shirley *et al*. 1995, Bharti and Khurana 1997). Their biosynthetic pathway, a branch of phenylpropanoid biosynthesis, has been extensively characterized. Two classes of genes are required for anthocyanin biosynthesis, the structural genes encoding the enzymes that directly participate in the formation of anthocyanins and other flavonoids, and the regulatory genes that control the transcription of structural genes (Quattrocchio *et al*. 1998, Grotewold *et al*. 2000, Davies *et al*. 2003).

There are numerous reports of the regulation of genes in the anthocyanin pathway by transcription factors, and collectively these have established that the components of the regulatory complex controlling anthocyanin biosynthesis are conserved in all higher plants (Holton and Cornish 1995). The anthocyanin pathway has been shown to be activated by a combination of R2R3 Myb, basic helix–loop–helix (bHLH) and WD40-type transcriptional factors similar in a wide variety of species, indicating that this function is well conserved (reviewed in Winkel-Shirley 2001, Koes *et al*. 2005). Several studies revealed that these Myb, bHLH, and WD40 proteins could interact physically,

indicating that they may operate in one transcription activation pathway and may activate their target genes as a (ternary) complex (Baudry *et al*. 2004, Carey *et al*. 2004, Morita *et al*. 2006, Goff *et al*. 1992, Gonzalez *et al*. 2008, Quattrocchio *et al*. 1999, Schwinn *et al*. 2006, Spelt *et al*. 2000, Taylor and Briggs 1990, de Vetten *et al*. 1997, Walker *et al*. 1999, Zhang *et al*. 2003, Zimmermann *et al*. 2004).

In higher plants, MYB transcription factors are encoded by super gene family, which is classified into three subfamilies, Myb1R, R2R3Myb and Myb3R, depending on the number of adjacent repeats in the MYB domain. The MYB domain is a region of about 52 amino acids that binds DNA in a sequence-specific manner. MYB transcription factors are represented by over one hundred members in the model plant Arabidopsis (Riechmann *et al*. 2000). MYB proteins with two repeats (R2R3-MYBs) are specific to plants and yeast (Jin and Martin, 1999) and are the most abundant type in plants (Riechmann *et al*. 2000). R2R3-MYBs are known to play important roles in regulating anthocyanin biosynthesis (Martin and Paz-Ares 1997, Zhang *et al*. 2000, Nesi *et al*. 2001).

The basic helix–loop–helix (bHLH) family of transcription factors is among the largest in animals and plants (Riechmann *et al*. 2000). bHLH family members have a basic helix–loop–helix domain that was initially identified in the animal transcriptional regulators MyoD and Myc (Murre *et al*. 1989).

WD40 repeat proteins comprise a family in the *β*-propeller protein group, which is characterized by the presence of a 40 residue core region delineated by a glycine–histidine (GH) dipeptide and a tryptophan–aspartate (WD) dipeptide (Smith *et al.* 1999). The WD40 repeat proteins form an ancient family that is found in all eukaryote genomes

analyzed. They have been reported from animals, fungi and the slime mould *Dictyostelium*, as well as from plants (Ramsay and Glover 2005).

A general Myb/bHLH/WD-repeat model for regulation of the anthocyanin biosynthetic pathway was found to operate in all plant species studied including snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*) and *Arabidopsis thaliana* (Morita *et al*. 2006, Quattrocchio *et al*. 1999, Schwinn *et al*. 2006, Spelt *et al*. 2000, de Vetten *et al*. 1997, Walker *et al*. 1999, Zhang *et al*. 2003).

Anthocyanin biosynthesis

Anthocyanins are synthesized in the cytoplasm, likely by a multienzyme complex anchored on endoplasmic reticulum (Winkel-Shirley 1999 and 2004). Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate and 4-coumarate:CoA ligase (4CL) converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3β-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins),

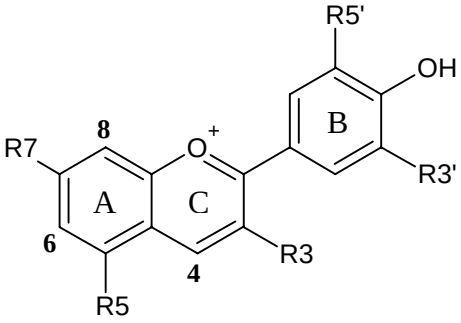
which are converted to anthocyanidins by anthocyanidin synthase (ANS),or also called leucoanthocyanidin dioxygenase (LDOX). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT), which stabilized the anthocyanidins by 3-O-glucosylation.

Proanthocyanidins are derived from the pathway leading to anthocyanins (Winkel-Shirley 2001). Three enzymes; leucoanthocyanidin reductase (LAR), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR); function at branches between anthocyanin and proanthocyanin biosynthesis. Synthesis of PA polymers is believed to occur by addition of an intermediate derived from a flavan-3,4-diol (such as leucocyanidin) to a flavan-3-ol terminal unit (such as catechin or epicatechin) with sequential addition of further extension subunits as the polymer lengthens. The anthocyanin and proanthocyanidin biosynthesis pathway is shown in Figure 3.

Anthocyanin transport to vacuole

Flavonoid glycosides, including anthocyanins, are usually transported into the vacuole. The transport mechanism is less well understood than the biosynthesis. Transport mechanisms may be redundant or depend on plant species and organs. The first and most established mechanism involves transport of anthocyanins via a glutathione *S*-transferase (GST)-like protein and a multi-drug resistance-like protein (a type of ABC transporter). Involvement of the former has been shown in maize (Marrs *et al*. 1995), petunia (Alfenito *et al*. 1998) and *Arabidopsis* (Kitamura *et al*. 2004), and the latter has been identified in maize (Goodman *et al*. 2004).

The anthocyanins and proanthocyanidins are transported from the cytoplasm into the vacuole, an acidic environment in which anthocyanins can accumulate to high levels, and in which they assume a brightly colored chemical structure (Kitamura 2006).



R3=R5=R7=OH		
Pelargonidin	R3′=H	R5′=H
Cyanidin	R3′=OH	R5′=H
Peonidin	R3′=OCH_3	R5′=H
Delphinidin	R3′=OH	R5′=OH
Petunidin	R3′=OCH_3	R5′=OH
Malvidin	R3′=OCH_3	R5′=OCH_3

Figure 1 Structures of major anthocyanidins

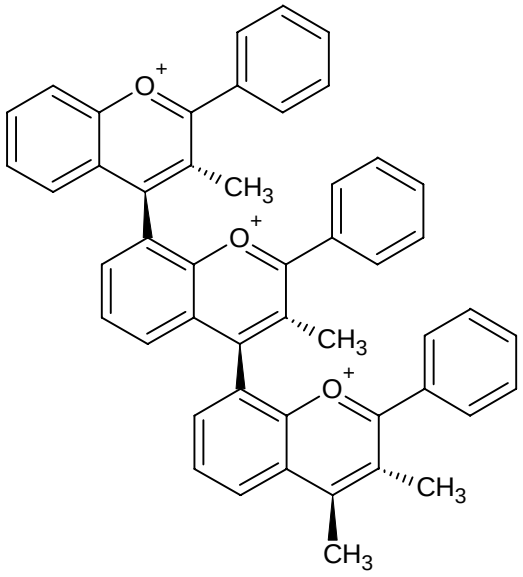


Figure 2 Basic structures of proanthocyanidins

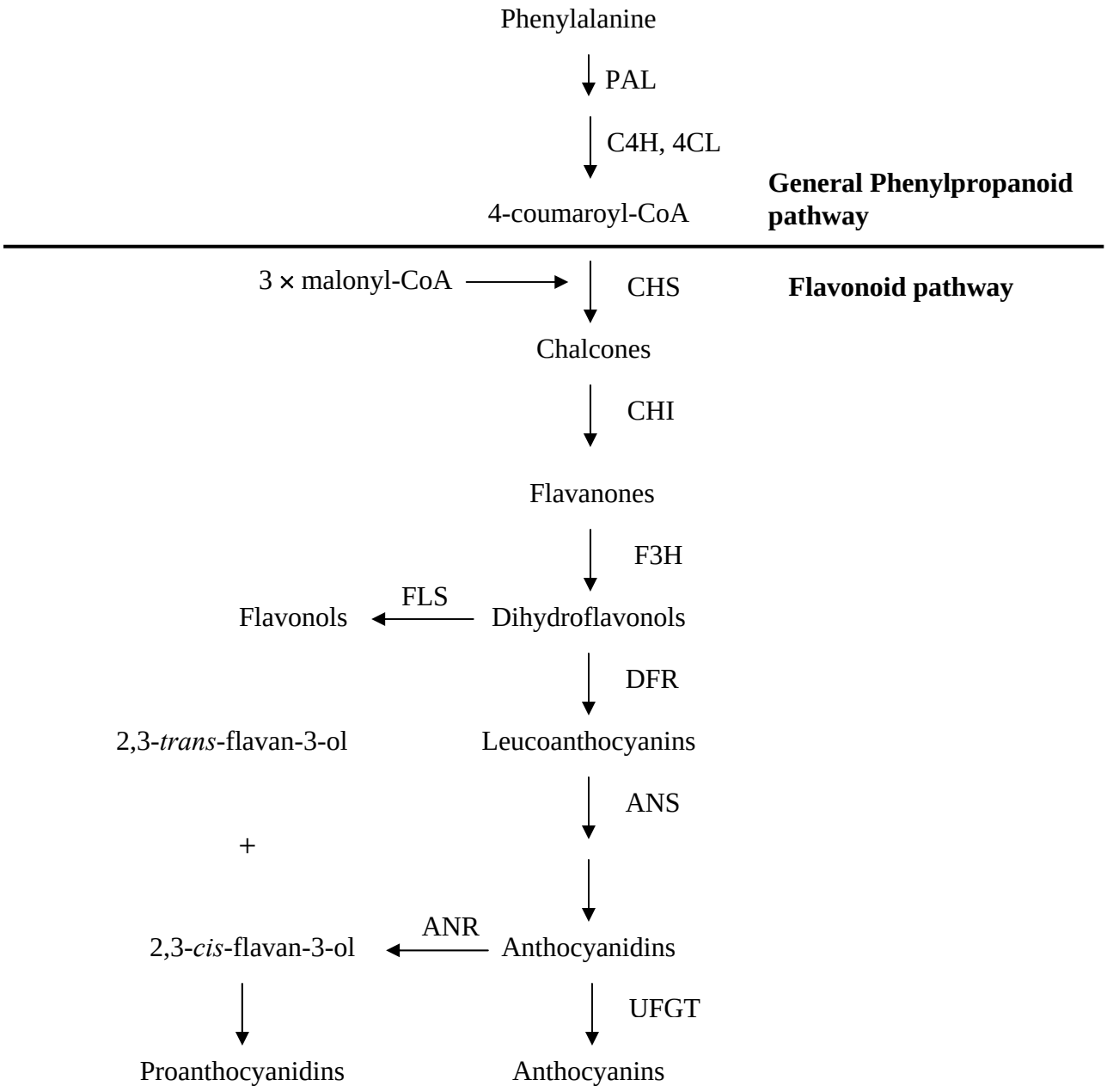


Figure 3 Schematic representation of the biosynthetic pathway for anthocyanins and proanthocyanidins. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, UDP glucose-flavonoid 3-o-glucosyl transferase.

CHAPTER ONE

CHAPTER ONE

Characterization of glutathione *S*-transferase (GST) genes upregulated by *PAP1* in *Arabidopsis thaliana*

1.1 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 (Droog *et al*. 1995). Glutathione *S*-transferases (GSTs) are found in plants, animals, fungi, and some bacteria, yet their role in plant biology is poorly understood. Most GSTs catalyze the conjugation of glutathione (GSH) to a variety of electrophilic substrates. However, some GSTs can function as GSH peroxidases and ligandins, making it difficult to assign specific roles for individual GST enzymes (Edwards *et al*. 2000). There have been studied that revealed the diversity of the GST super family in higher plants (McGonigle *et al*. 2000, Wagner *et al*. 2002).

GSTs, originally characterized by their ability to modify xenobiotics covalently by glutathionation, are rapidly induced by their substrates such as plant herbicides (Marrs *et al*. 1995). In addition, GSTs act as non-enzymatic carrier proteins (ligandins) enabling intracellular shuttling of endogenous compounds in animals, plants, and prokaryotes (Marrs *et al*. 1995, Mueller *et al*. 2000).

Anthocyanins and proanthocyanidins are localized in the vacuole; however, the enzymatic steps of their synthesis occur in the cytoplasm (Mol *et al*. 1998, Springob *et al*. 2003). GST plays a role in the vacuolar transport of anthocyanins, and it is represented by Bz2 in maize (Marrs *et al*. 1995) and AN9 in petunia (Alfenito *et al*. 1998). GSTs presumably act as flavonoid-binding proteins and play the role of cytoplasmic flavonoid-carrier proteins *in vivo* (Mueller *et al*. 2000). In *Arabidopsis thaliana*, *TRANSPARENT TESTA 19* (*TT19*, At5g17220), a gene encoding a GST, has recently been isolated. TT19

is required for the accumulation of anthocyanin in vegetative tissues and proanthocyanidin in the seed coat (Kitamura *et al*. 2004).

GSTs constitute a family of multifunctional enzymes present in both plants and animals (Edwards *et al*. 2000, Frova 2003). The main function of GSTs is to detoxify xenobiotics (Frova 2003). GSTs also play an indispensable role in the intracellular transportation of anthocyanins and proanthocyanidins (Marrs *et al*. 1995, Alfenito *et al*. 1998, Mueller *et al*. 2000, Cho and Kong 2003, Kitamura *et al*. 2004). Based on gene organization (intron number and position), sequence similarity, and the conservation of specific residues, plant GSTs can be classified into the following 5 classes: zeta, theta, tau, phi, and lambda (Dixon *et al*. 2002a). Recently, a new group of GST-like proteins with glutathione-dependent dehydroascorbate reductase (DHAR) activity has been classified in *Arabidopsis* and some other plant species (Dixon *et al*. 2002b, Frova 2003).

The *pap1-D* mutant is a transferred DNA (T-DNA) activation-tagged line that overproduces anthocyanins by the ectopic overexpression of the *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*) gene encoding an MYB transcriptional factor. This overexpression occurs by the action of tetramerized enhancer sequences present in the cauliflower mosaic virus 35S promoter in the inserted T-DNA (Borevitz *et al*. 2000). Overexpression of the *PAP1* gene of *Arabidopsis* activates most of the genes in the anthocyanin pathway, leading to anthocyanins production throughout the plant (Borevitz *et al*. 2001, Tohge *et al*. 2005). Thus, it is a potential tool for the functional identification of genes involved in anthocyanin biosynthesis. In *PAP1*-overexpressing mutant plants, 3 members of the *GST* gene family—*TT19*, *AtGSTF5* (At1g02940), and *AtGSTF6* (At1g02930)—were found to be up-regulated 19.1-, 3.6-, and 17.9-fold, respectively

(Tohge *et al*. 2005). In this study, the enzymatic activities of recombinant proteins and the metabolite changes in knockout mutants of these *GST*-like genes were examined.

1.2 Results

1.2.1 Phylogenetic tree Phylogenetic analysis of *Arabidopsis* GST homologues

Phylogenetic analysis of the deduced amino acid sequences of *Arabidopsis* GSTs and known GSTs that are involved in anthocyanin biosynthesis were showed in Figure 4. The phylogenetic tree showed that TT19, *At*GSTF5, and *At*GSTF6 were classified in the phi class, as were AN9 from petunia (*Petunia hybrida*) and *Pf*GST1 from perilla (*Perilla frutescens*); in contrast, Bz2 from maize (*Zea mays*) was classified into the tau class.

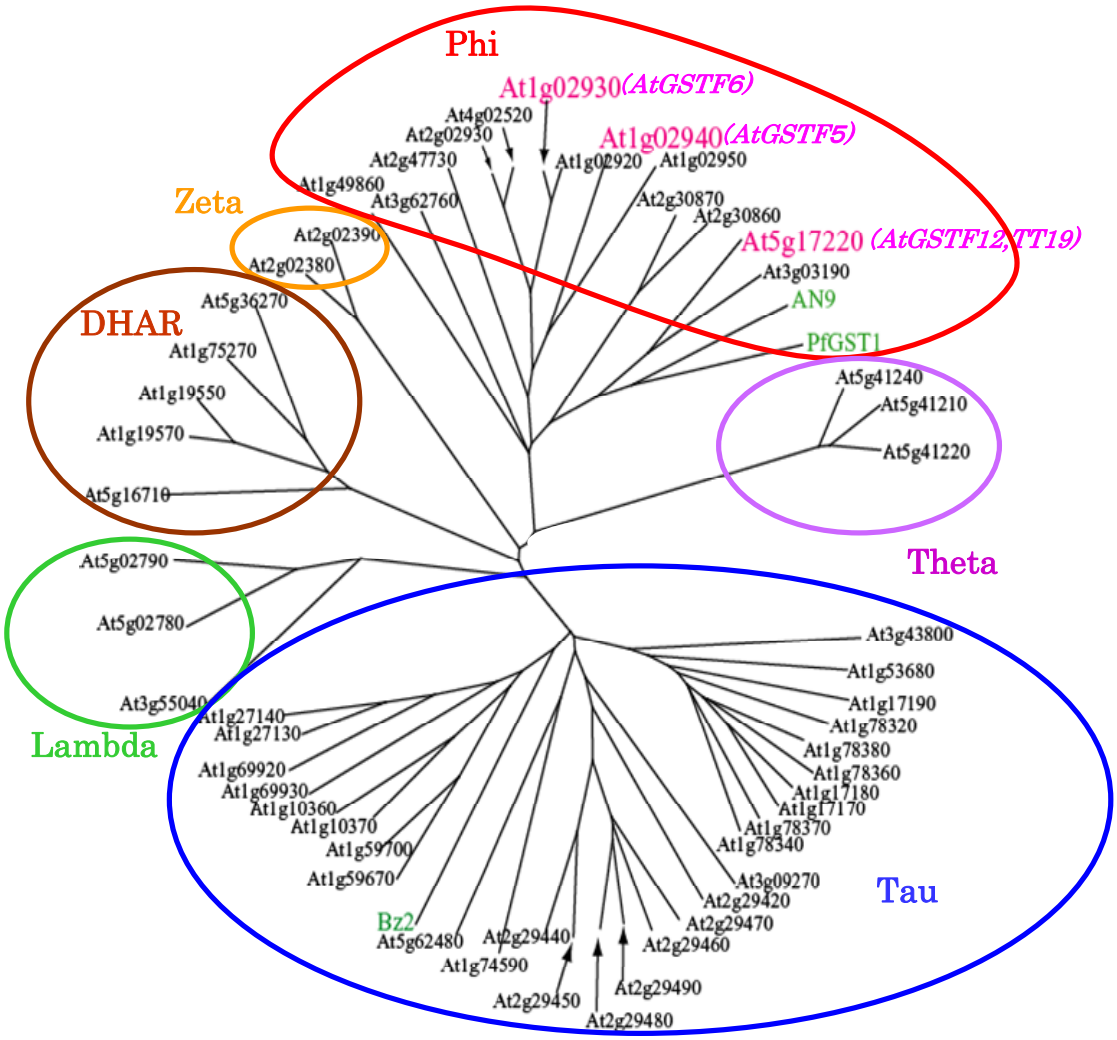


Figure 4 Phylogenetic analysis of *Arabidopsis* GST homologues

Phylogenetic analysis of *Arabidopsis* GST-like proteins using amino acid sequences from GenBank database presented in the *Arabidopsis* genome initiative (AGI) code. Phylogenetic tree was constructed using the ClustalW program with the neighbor-joining method (Thompson *et al*. 1994) and TreeView X. The reported GSTs—AN9 from petunia (*Petunia hybrida*) (Alfenito *et al*. 1998), *Pf*GST1 from perilla (*Perilla frutescens*) (accession number AB362191), and Bz2 from maize (*Zea mays*) (Marrs *et al*. 1995)—were also included.

1.2.2 Expression in *E. coli* and purification of recombinant GST protein

To express the recombinant proteins, the cDNAs of *TT19*, *AtGSTF5*, and *AtGSTF6* were introduced into the Gateway™ system (Invitrogen Corp., CA, USA) following the manufacturer's instructions. Entry clones were then obtained by BP recombination with pDONR221. The nucleotide sequences of the entry clones were determined to confirm the sequence. The cDNA coding region for the GST proteins were cloned into bacteria expression pDEST17 expression vector, that contains N-terminal 6×histidine (6×His) tag. The expression vectors were expressed in *Escherichia coli* BL21 AI™. The bacteria harboring this expression plasmid were induced with 0.2% L-arabinose at a temperature of 20 °C and produced the recombinant GST proteins. The recombinant of TT19 is 26 kD, *At*GSTF6 is 24 kDa and *At*GSTF5 is 31 kDa. The recombinant proteins were purified by using nickel affinity chromatography. The protein levels were analyzed using the dye-binding method (Bio-Rad, CA, USA); sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using 12% polyacrylamide gels, and Coomassie brilliant blue staining was performed (Figure 5).

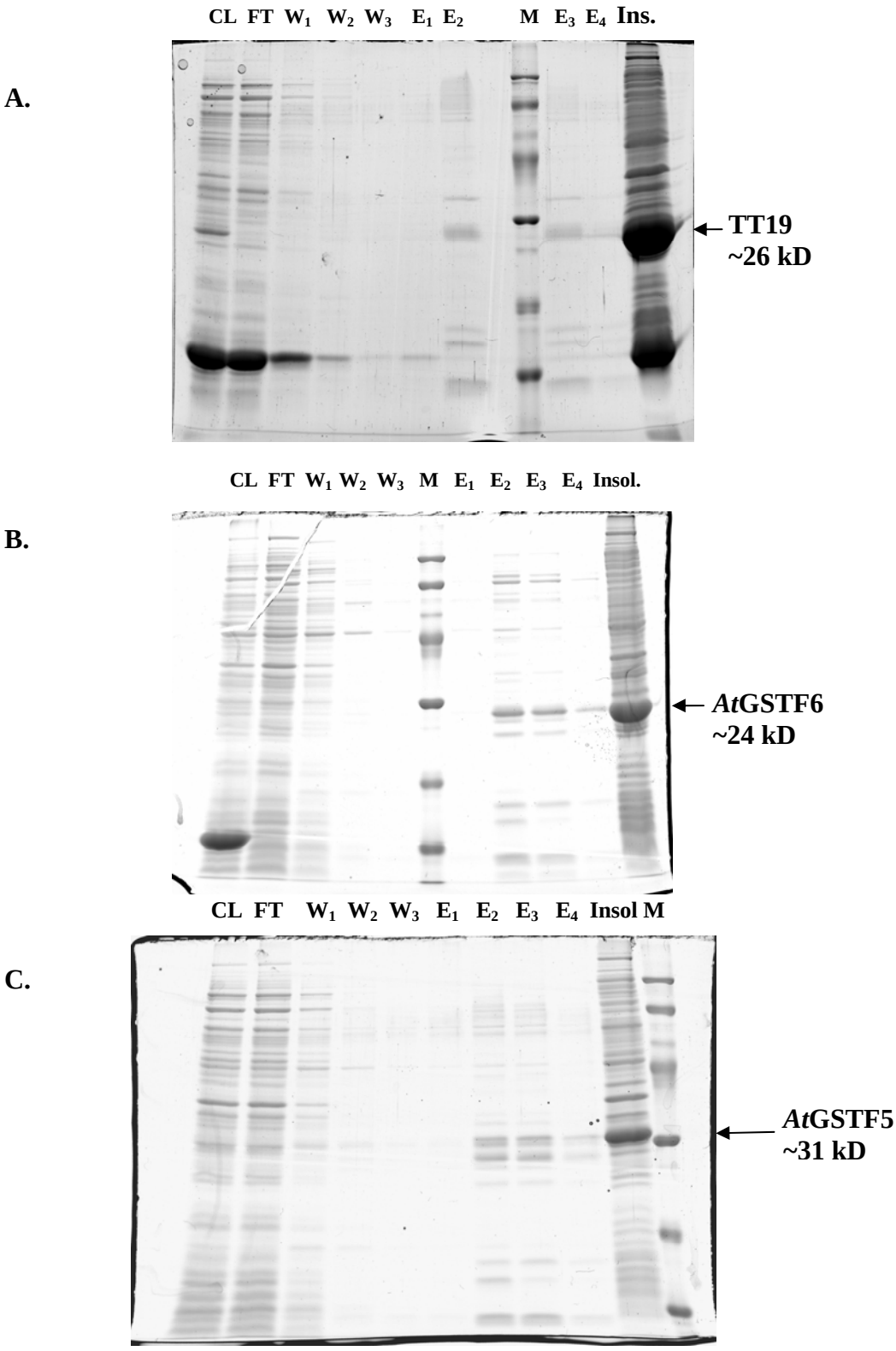


Figure 5 Expression and purification of recombinant GST proteins

A. TT19 **B.** *At*GSTF6 **C.** *At*GSTF5, CL = clear lysate, FT = flow through, W1 = first wash, W2 = second wash, W3 = third wash, E1 = first elution, E2 = second elution, E3 = third elution, E4 = forth elution, M = low molecular marker, Ins. = insoluble fraction

1.2.3 *In vitro* GST enzymatic activity assay

The GST activities of the purified proteins, i.e., their ability to conjugate glutathione the universal substrate 1-chloro-2,4-dinitrobenzene (CDNB) were determined. CDNB is usually used as a model GST substrate (Edward *et al*. 2000). The absorbance at 340 nm was used to measure the amount of the conjugated product, i.e., dinitrophenol-glutathione (DNP-GS; Marrs 1996), at 25 °C in a reaction buffer containing 98 mM potassium phosphate (pH 6.5), 0.98 mM EDTA (Habig *et al.* 1974), 2 mM reduced glutathione (Wako, Osaka, Japan), and 1 mM CDNB (Wako, Osaka, Japan). GST from rat liver (Sigma-Aldrich, St. Louis, USA) was used as positive control.

As shown in Table 1, all the recombinant proteins of the *Arabidopsis* GST-like genes exhibited GST activity, although the activity was very weak compared with that of the authentic rat liver GST. On comparison with the Bz2 and AN9 recombinant proteins expressed in *E. coli*, it was found that the recombinant proteins of the *Arabidopsis* GST-like genes showed a similar level of the GST activity of CDNB conjugation as Bz2 (Marrs *et al*. 1995). However, AN9 showed several-fold higher GST-specific activity than *Arabidopsis* proteins (Alfenito *et al.* 1998).

Table 1 GST activity of purified TT19, *At*GSTF5 and *At*GSTF6 recombinant proteins against CDNB, values are means ± SD (n = 3)

Proteins	GST specific activity (nmol min^{-1} μg^{-1} protein)
Standard GST from rat liver	383.1 ± 0.1
TT19	0.52 ± 0.02
*At*GSTF5	0.36 ± 0.02
*At*GSTF6	0.87 ± 0.05

1.2.4 GST genes transcripts in mutant plants

Salk_105779 (designated as *gstf6-1*), an *A. thaliana* (ecotype Columbia) T-DNA insertion mutant of *AtGSTF6* and Salk_026398 (designated as *gstf12-1*), an insertion mutant of *TT19*, were obtained from the Salk Institute collection (Alonso et al. 2003). To confirm the T-DNA insertion and determine its position in the *gstf12-1* and *gstf6-1* lines, the genomic DNAs extracted from the leaves were polymerase chain reaction (PCR)-amplified with a combination of specific primers designed for the individual lines and LBb1. The line *gstf12-1* contained a T-DNA insertion at the second intron of At5g17220 (*TT19*), and the line *gstf6-1* had a T-DNA insertion at the first intron of At1g02930 (*AtGSTF6*) (Figure 6A).

The levels of *TT19* and *AtGSTF6* gene transcripts in the homozygotes of the T-DNA-inserted mutants were determined by semiquantitative reverse transcription (RT)-PCR using gene-specific primers in 3-week-old wild type plants and the mutants. The transcripts of *AtGSTF6* and *TT19* were not observed in the homozygotes of the *gstf6-1* and *gstf12-1* mutants, respectively, while they were in the wild-type plant (Figure 6B). These finding suggest that T-DNA insertion knockout mutants were obtained.

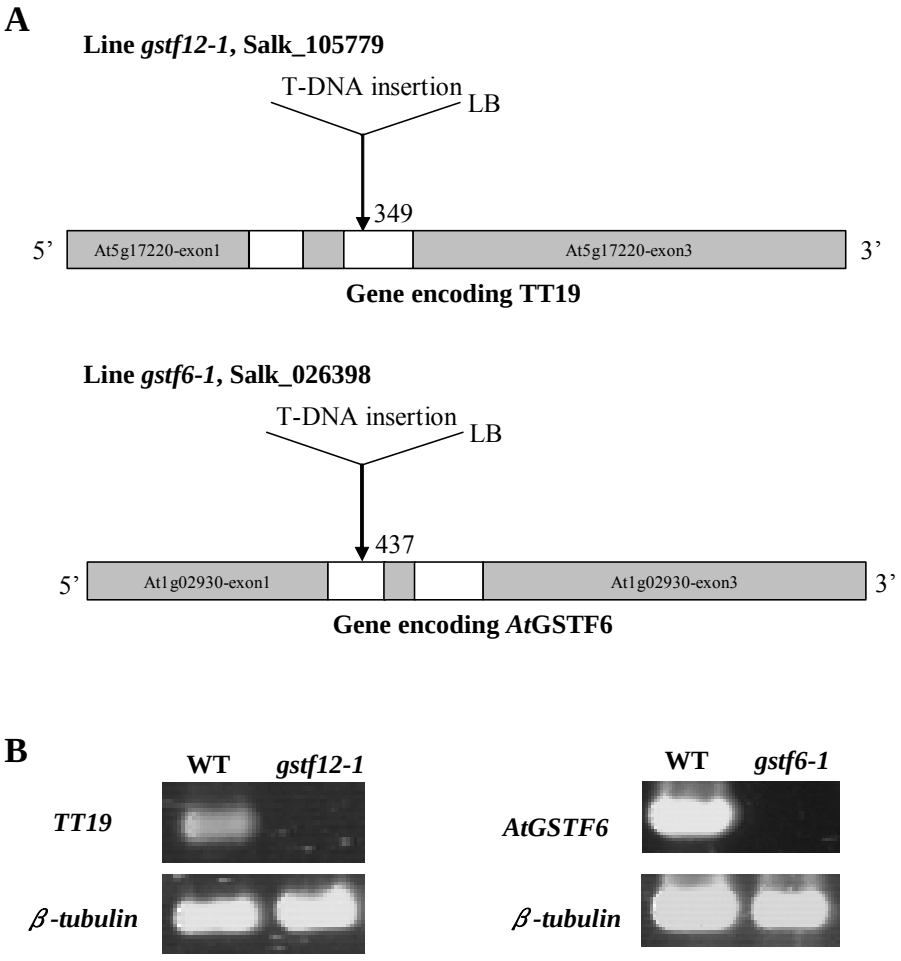


Figure 6 T-DNA-inserted mutants of *TT19* (At5g17220) and *AtGSTF6* (At1g02930)

A. Schematic structure of the T-DNA-inserted lines of *TT19* (At5g17220) (line *gstf12-1*, Salk_105779) and *AtGSTF6* (At1g02930) (line *gstf6-1*, Salk_026398).

B. Expression of the *TT19* and *AtGSTF6* genes in mutant plants compared with that in wild-type Col-0 (WT) plant. Total RNA was isolated from the rosette leaves of 3-week-old plants grown under the high-sugar stress condition (10% sucrose). β-Tubulin was used for normalization. (left) *gstf12-1* (Salk_150779) mutant. (right) *gstf6-1* (Salk_026398) mutant.

1.2.5 Flavonoids analysis in GST knockout mutants

The T-DNA-inserted mutants of *TT19* (Salk_105779, *gstf12-1*) and *AtGSTF6* (Salk_026398, *gstf6-1*) were analyzed in terms of flavonoid and anthocyanin accumulation. For flavonoid analysis, the rosette leaves of the 3-week-old plants were harvested and extracted with 5 μl extraction solvent (methanol:glacial acetic acid:water = 45:5:50) per milligram fresh weight of tissue. The extracts were analyzed by using a high-performance liquid chromatography/photodiode array detection/electrospray ionization mass spectrometry (HPLC/PDA/ESI-MS) system. The accumulation of anthocyanins in the *gstf12-1* mutant was severely decreased (96%) compared with that in the wild-type plants. In contrast, both the total anthocyanin level and the anthocyanin pattern of the *gstf6-1* mutant showed no significant change. With regard to the level and composition of flavonoids, both the *gstf6-1* and *gstf12-1* mutants showed no obvious change compared to the wild-type control plants (Figure 7A, B). The structures of anthocyanins (cyanidin derivatives A1-A4) and flavonoids (Kaempferol glycosides F1-F3) accumulated *Arabidopsis thaliana* as shown in figure 7A; A1, cyanidin 3-*O*-[2"-*O*-(xylosyl) 6"-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-[6"'-*O*-(malonyl) glucoside]; A2, cyanidin 3-*O*-[2"-*O*-(xylosyl) 6"-*O*-(*p*-coumaroyl) glucoside] 5-*O*-[6"'-*O*-(malonyl) glucoside]; A3, cyanidin 3-*O*-[2"-*O*-(2"'-*O*-(sinapoyl) xylosyl) 6"-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-glucoside; A4, cyanidin 3-*O*-[2"-*O*-(2"'-*O*-(sinapoyl) xylosyl) 6"-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-[(6""-*O*-malonyl) glucoside]; F1, kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside; F2, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside; F3, kaempferol 3-*O*-[6"-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; are shown in Figure 8.

These results suggest that *TT19* is almost exclusively involved in anthocyanin accumulation; the mutation in *TT19* thus causes a change in the anthocyanin accumulation level, and no other genes including *AtGSTF6* and possibly *AtGSTF5*, can complement.

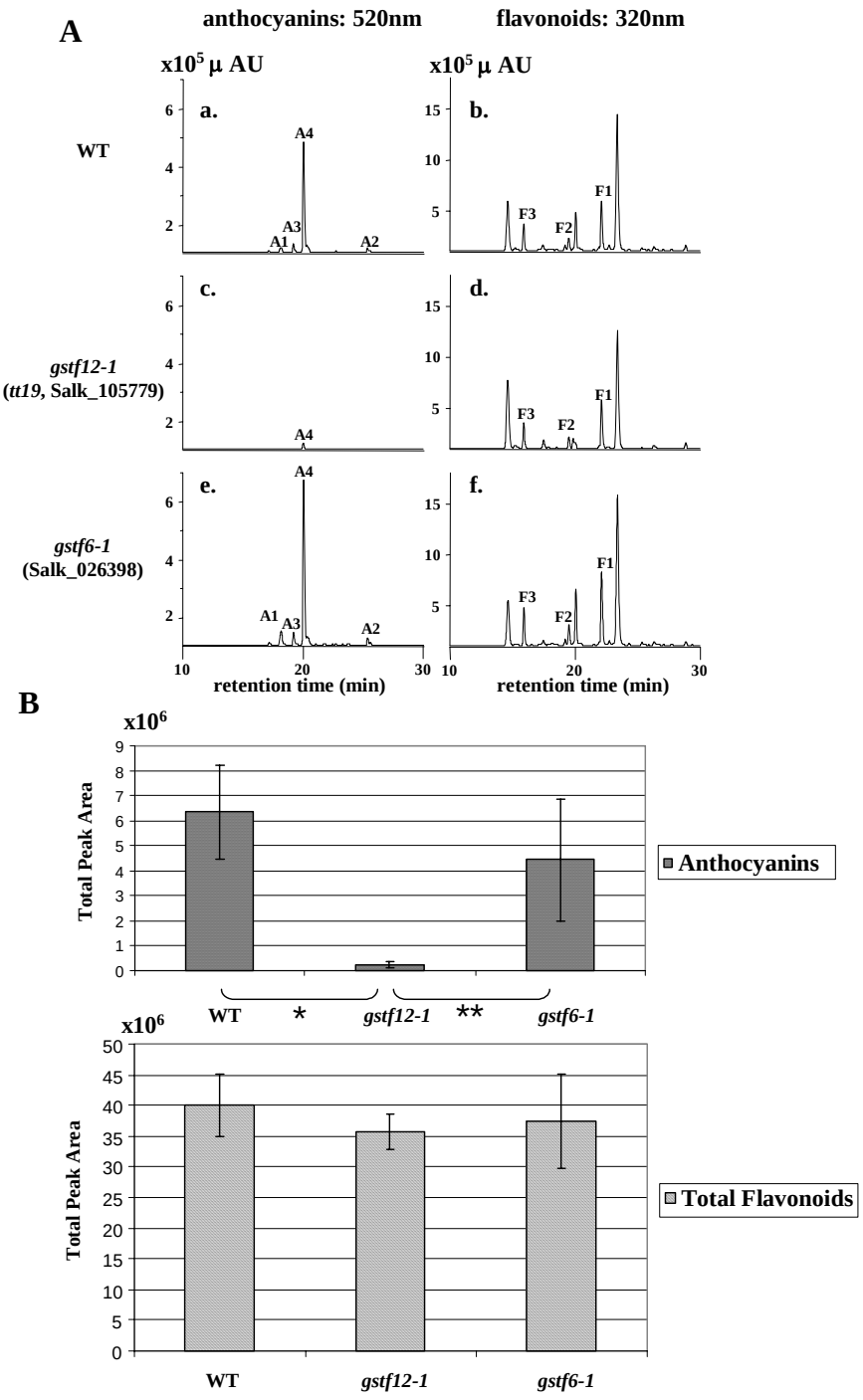
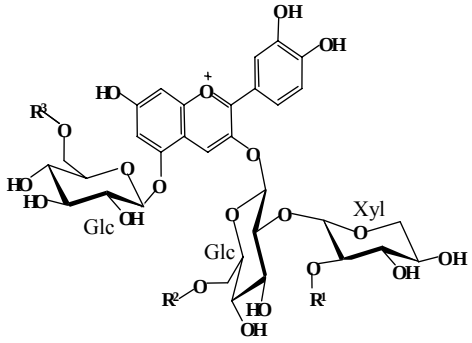


Figure 7 Flavonoid accumulation in knockout mutants

A. HPLC/PDA chromatograms of rosette leaf extract from wild-type (WT) *Arabidopsis*, the *tt19* knockout mutant (*gstf12-1*), and the *Atgstf6* knockout mutant (*gstf6-1*). (a,c,e) Absorbance at 520 nm for anthocyanin detection (b,d,f) Absorbance at 320 nm for flavonoid detection

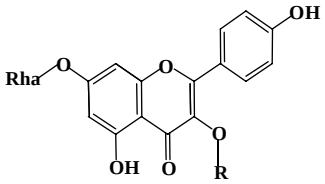
B. Total peak area analyzed from liquid chromatography/mass spectrometry (LC-MS) data of wild-type (WT) *Arabidopsis*, the *tt19* knockout mutant (*gstf12*-1), and the *Atgstf6* knockout mutant (*gstf6-1*). The values represent the mean with standard deviation (SD) of triplicate determination. Data were analyzed by one-way analysis of variance as indicated with Bonferroni *post hoc* adjustment for multiple testing (*; $p < 0.05$, **; $p < 0.005$).

cyanidin derivatives



A1: R^1 = -H, R^2 = -*p*-coumaroyl-Glc, R^3 = -malonyl
A2: R^1 = -sinapoyl, R^2 = -*p*-coumaroyl, R^3 = -malonyl
A3: R^1 = -sinapoyl, R^2 = -*p*-coumaroyl-Glc, R^3 = -H
A4: R^1 = -sinapoyl, R^2 = -*p*-coumaroyl-Glc, R^3 = -malonyl

kaempferol glycosides



F1: R=-Rha
F2: R=-Glc
F3: R=-Glc-Rha

Figure 8 Structures of anthocyanins and flavonoids accumulated in *Arabidopsis thaliana*

1.2.6 Network analysis of co-expression among flavonoid related genes

The co-expression networks among 39 genes up-regulated by *PAP1* (Tohge *et al.* 2005) and all *Arabidopsis* genes were evaluated using the ATTED-II database (*Arabidopsis thaliana* trans-factor and cis-element prediction database), which allows co-expression analyses based on publicly available transcriptome data via a correlated gene search program available at the RIKEN PRIMe website (http://prime.psc.riken.jp/). The linkages between genes that had a higher correlation coefficient ($r > 0.6$) in 3 data sets; all data set version 1 (771 data), tissue and development version 1 (237 data), and stress treatment version 1 (298 data). The co-expression networks were computed using 39 *PAP1*-up-regulated genes as "'bait" or "guide". As shown in Figure 9, the networks were divided into 4 groups: anthocyanin biosynthesis, proanthocyanin biosynthesis, general flavonoid biosynthesis, and lignin biosynthesis. These networks indicated that *TT19* is tightly connected to anthocyanin biosynthesis; however, in contrast, *AtGSTF5* and *AtGSTF6* showed no connection to anthocyanin biosynthesis. *AtGSTF5* and *AtGSTF6* exhibited co-expression with *TT2* (R2R3Myb) and At1g05575 (unknown), respectively. These results suggest that the up-regulated genes in the *PAP1*-overexpressing mutant are not directly involved in anthocyanin accumulation.

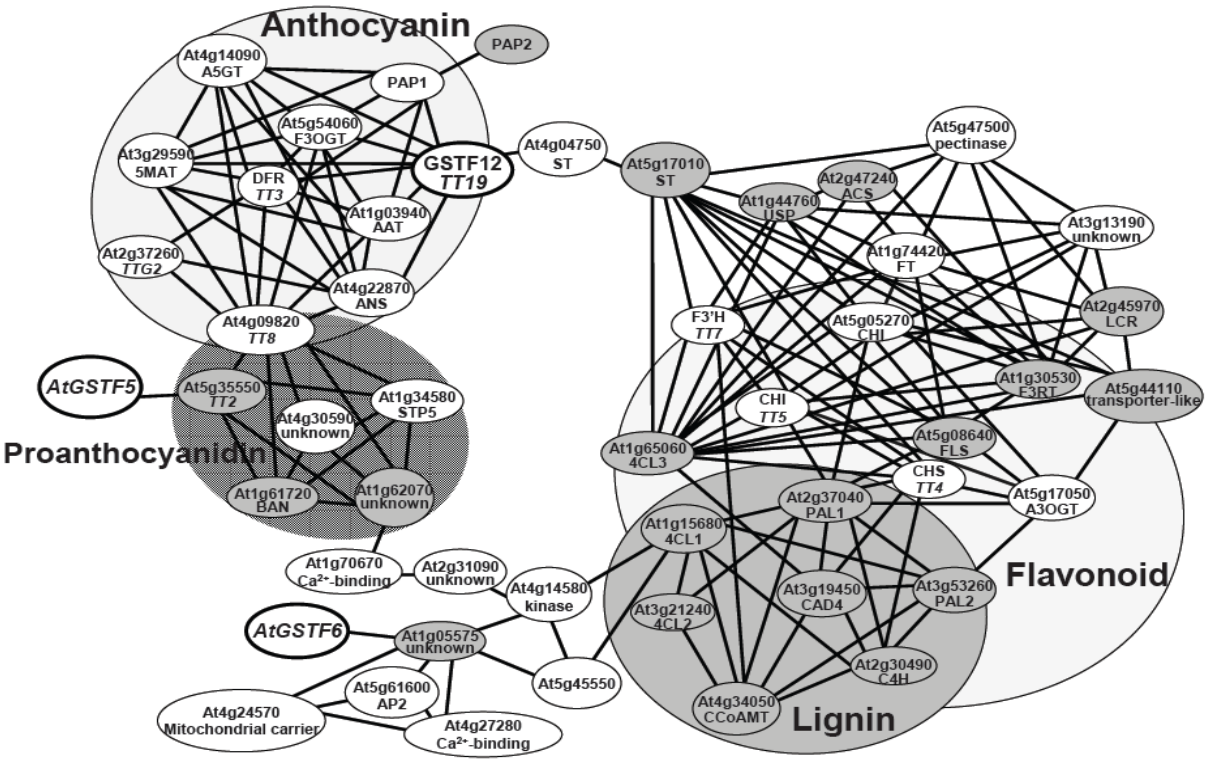


Figure 9 Co-expression networks of *PAP1*-upregulated genes with whole genes

Co-expression analysis was performed using the "Correlated Gene" program available at RIKEN PRIMe (http://prime.psc.riken.jp/) based on ATTED-II, and the results were drawn using the Pajek program. The genes up-regulated by *PAP1* are indicated by white cycles. Positive correlations ($r > 0.6$) in all data sets are indicated by connecting lines. A5GT, anthocyanidin 5-*O*-glucosyltransferase; PAP, production of anthocyanin pigment; F3OGT, flavonoid 3-*O*-glucosyltransferase; 5MAT, 5-*O*-malonyltransferase; DFR, dihydroflavonol 4-reductase; TT, transparent testa; AAT, anthocyanin acyltransferase; TTG, transparent testa glabra; ANS, anthocyanidin synthase; STP, monosaccharide transporter protein; BAN, BANYULS; AP2, activator protein 2; 4CL, 4-coumarate:CoA ligase; CCoAAMT, caffeoyl-CoA *O*-methyltransferase; PAL, phenylalanine ammonia-lyase; CAD4, cinnamyl alcohol dehydrogenase 4; C4H, cinnamate-4-hydroxylase; CHS, chalcone synthase; A3OGT, anthocyanidin 3-*O*-glucosyltransferase; FLS, flavonol synthase; CHI, chalcone isomerase; F3RT, flavonol-3-*O*-rhamnosyltransferase; LCR, lacerate; F3'H, flavonoid 3' hydroxylase; FT, fucosyltransferase; USP, universal stress protein; ACS, acyl-CoA synthetase; ST, sugar transporter.

1.3 Discussion

In vitro GST enzymatic activity assay

The results of *in vitro* GST enzymatic activity assay using the purified recombinant proteins expressed in *E.coli* BL21AI™ exhibited GST activity in all recombinant proteins tested as shown in Table 1, although the activity was very weak compared with that of the authentic rat liver GST. On comparison with the Bz2 and AN9 recombinant proteins expressed in *E. coli*, it was found that the recombinant proteins of the *Arabidopsis* GST-like genes showed a similar level of the GST activity of CDNB conjugation as Bz2 (Marrs *et al*. 1995). However, AN9 showed several-fold higher GST-specific activity than *Arabidopsis* proteins (Alfenito *et al*. 1998).

From the very weak activity of all recombinant proteins showed that the recombinant GST from plants are not acting like enzymes for catalyze the conjugation of GSH and CDNB.

GSTs are multifunctional enzymes involved in vacuolar sequestration by conjugation with glutathione (Edwards *et al.* 2000, Dixon *et al.* 2002). However, anthocyanin-glutathione has not been identified in plant extracts. The vacuolar sequestration of anthocyanin depends on defined GSTs, AN9 from petunia (Alfenito *et al.* 1998), but GSH conjugation does not occur either (Mueller *et al*. 2000). Recently, TT19 is known to require for efficient vacuolar sequestration of anthocyanin in *Arabidopsis thaliana* (Kitamura *et al*. 2004) and was confirmed in the experiment of anthocyanin accumulation in knockout mutants (Figure 7). These results suggest that TT19 might be acting as cytoplamic escort or carrier protein rather than glutathionation agent.

Flavonoids analysis in GST knockout mutants

In the homozygous *gstf12-1* mutant, the total anthocyanin was severely decreased to 96% of that in the wild type, although the composition of the accumulated flavonoids was the same as in the wild type. From the results suggest that *TT19* is involved in anthocyanin accumulation but seem to be not involved in flavonoid accumulation in rosette leave. The flavonoid accumulation may be not required GSTs for transport. There have been the hypothesis that flavonoids are synthesized in the same cells in which they accumulate and there have been the report support the hypothesis (Peer *et al.* 2001).

Both the total anthocyanin level and the anthocyanin pattern of the *gstf6-1* mutant showed no significant change. *AtGSTF6* might not be involved in anthocyanin biosynthesis but might be involved in transportation of other compounds accumulated in *PAP1*-upregulated mutant.

Network analysis of co-expression among flavonoid related genes

Gene expression microarrays provide a revolutionary approach for measuring the mRNA levels of thousands of genes at the same time. Systematic analysis of genome-wide expression profiles across multiple conditions are useful system for study the biological networks.

Because genes that encode proteins that participate in the same pathway or are part of the same protein complex are often co-regulated, clusters of genes with related functions often exhibit expression patterns that are correlated under a large number of diverse conditions in DNA microarray experiments (Stuart *et al.* 2003).

The co-expression data from DNA microarray in each experiment, such as tissue and development or stress treatment, were plot based on Pearson correlation. The correlation coefficient or r-value of gene pairs was calculated (Quackenbush 2001).

From the network analysis of co-expression results, *TT19* is tightly connected to anthocyanin biosynthesis; however, in contrast, *AtGSTF5* and *AtGSTF6* showed no connection to anthocyanin biosynthesis. The results corresponded to the proposition of the predominant role of *TT19* in anthocyanin accumulation.

CHAPTER TWO

CHAPTER TWO

Protein interaction among the regulatory factors involved in anthocyanin biosynthesis in *Perilla frutescens*

2.1 Introduction

Perilla frutescen Britton var. *crispa* Thuab), or Shiso in Japanese, belongs to family Labiatae (or Lamiaceae). Two cultivars of *P. frutescens*, red (Aka-jiso) and green (Ao-jiso) forms, are known to differ with regards to anthocyanin accumulation (Figure 10). Red/purple leaves of Aka-jiso which contains high amounts of anthocyanin, have been traditionally used as a food colorant and medicine in Japan, China and other Asian countries for a long time (Heci 2001). It has been shown that the red color is mainly due to the presence of malonylshisonin (3-*O*-(6-*O*-(*E*)-*p*-coumaryl-*β*-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)-cyanidin (Kondo *et al*. 1989, Meng *et al.* 2006) (Figure 11). There have been studied about perilla bioactivities, such as antioxidant (Jung *et al*. 2001), anti-allergic (Makino *et al*. 2003, Takano *et al*. 2004), anti-inflammatory (Ueda *et al*. 2002), and anti-HIV-1 activity (Yamasaki *et al*. 1998).

Structural genes encoding these enzymes involved in anthocyanin biosynthesis pathway of *P. frutescens* have been isolated and characterized. The expression of the structural genes in anthocyanin biosynthesis showed differential expression between red and green formas and also showed the light-induced manner expression suggested the presence of regulatory factor(s) that control the expression of all structural genes in the pathway (Gong *et al.* 1997).

Figure 10 *Perilla frutescen* var. *crispa*

Left Red forma or Aka-jiso, **Right** green forma or Ao-jiso

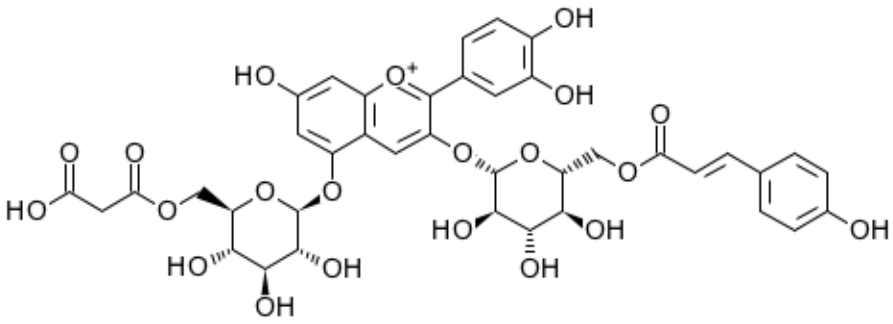


Figure 11 Structure of Malonylshisonin

cyanidin 3-*O*-(6’’-*O*-(*E*)-*p*-coumaryl)-β-D-glucopyranoside-5-*O*-[(6’’’-*O*-malonyl)-β-D-glucopyranoside]

There are numerous reports of the regulation of genes in the anthocyanin pathway by transcription factors, and collectively these have established that the components of the regulatory complex controlling anthocyanin biosynthesis are conserved in all higher plants (Holton and Cornish 1995). These transcriptional regulators include members of the Myb, bHLH and WD-repeat families (reviewed in Winkel-Shirley 2001, Koes *et al*. 2005). A general WD-repeat/Myb/bHLH model for regulation of the anthocyanin biosynthetic pathway was found to operate in all plant species studied including snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*) and *Arabidopsis thaliana* after two families of regulatory genes are found in maize, the *C1* and *R* families (Gonzalez *et al*. 2008). The members of *C1* family (*C1* and *PL*) encode MYB domain proteins (Cone *et al*. 1986), while the members of the *R* family (*R, LC, SN* and *B*) encode MYC proteins with a basic helix-loop-helix (bHLH) DNA binding motif (Goff *et al*. 1992). Several studies revealed that these MYB, BHLH, and WD40 proteins could interact physically, indicating that they may operate in one transcription activation pathway and may activate their target genes as a complex (Goff *et al*. 1992, Zhang *et al*. 2003, Baudry *et al*. 2004, Zimmermann *et al*. 2004).

The proposed regulatory factors of MYC and MYB homologues in *P. frutescens* (MYC-RP and MYB-P1, respectively) have been investigated (Gong *et al.*, 1999a, b). Genes identified to be responsible for anthocyanin biosynthesis are preferentially or exclusively expressed in anthocyanin producing tissues. The WD-repeat, *PfWD*, was isolated from red *P. frutescens* (Sompornpailin *et al*. 2002). Compared with other plants, several regulatory genes, which are presumably involved in anthocyanin biosynthesis in *Perilla* have been isolated and identified (unpublished data) as shown in Table 2.

Table 2 Regulator genes involved in anthocyanin biosynthesis isolated in many plants

Type	Maize	Petunia	Snapdragon	Arabidopsis	Perilla
MYB	*C1* *Pl* *P*	*AN2,* *AN4*	*ROSEA*	*PAP1* *TT2*	*MYB-P1* *MYB-C05*
MYC (bHLH)	*R* *B*	*JAF13* *AN1*	*DELILA*	*TT8*	*MYC-RP/GP* *MYC-F3G1* *MYC-RS*
WD40		*AN11*		*TTG1*	*PfWD*

Protein–protein interactions play crucial roles in the execution of various biological functions. Most proteins require physical interactions with other proteins to fulfill their biological role. Several commonly used protein-protein detection techniques have been described, including co-immunoprecipitation, glutathione-*S*-transferase (GST) pull-down experiments and yeast two-hybrid analyses (Walhout *et al.* 2000). The two-hybrid system is a molecular genetic tool which facilitates the study of protein-protein interactions (Ito *et al*. 2001). In the yeast two-hybrid system, a protein of interest, X, is fused to the DNA binding domain (DBD) of a transcription factor, such as Gal4p. The second hybrid protein, Y, is fused to a transcriptional activation domain (AD). A physical interaction between X and Y results in the reconstitution of a functional transcription factor that can activate expression of reporter genes. Usually, reporter genes that allow growth selection on specific media are used (Fields and Song 1989). However, the false positive is the large problem in yeast two-hybrid assay. The false positive can be reduce

by using low expression levels of the two hybrid protein and use of multiple reporter genes utilizing different promoters (Vidal and Legrain 1999). The scheme of yeast two-hydrid assay is shown in Figure 12. The yeast MaV203 used in this study MaV203 contains single copies of each of three reporter genes (*HIS3*, *URA3* and *lacZ*) that are stably integrated at different loci in the yeast genome. The promoter regions of *URA*3, *HIS*3, and *lacZ* are unrelated (except for the presence of GAL4 binding sites).

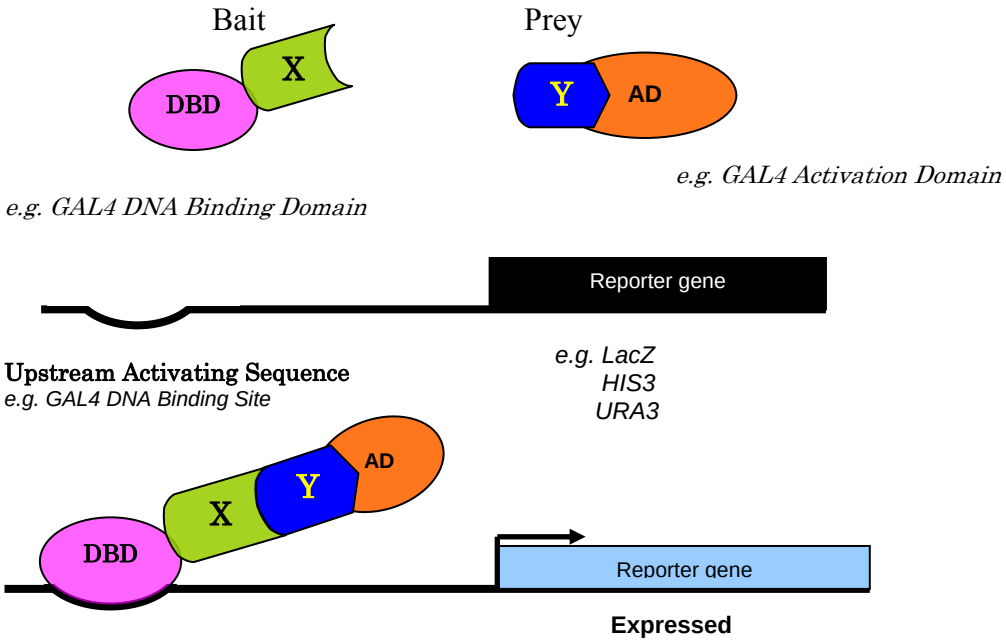


Figure 12 The scheme of yeast two-hybrid assay

Upper, Bait = DNA Binding Domain (DBD)/protein X fusion, Prey = Activation Domain (AD)/protein Y fusion.

Lower, when protein X interact with protein Y, the functional transcription activator will transcribe the reporter genes

In this study, four regulatory factor candidates isolated from *P. frutescens*, Myc-RP, Myc-RS, Myb-C05 and *Pf*WD were studied the protein-protein interaction using yeast two-hybrid system. The ProQuest™ Two-Hybrid System (Invitrogen Corp., Carlsbad, CA, USA) was used. The vectors of bait and prey used in this study are shown in Figure 13.

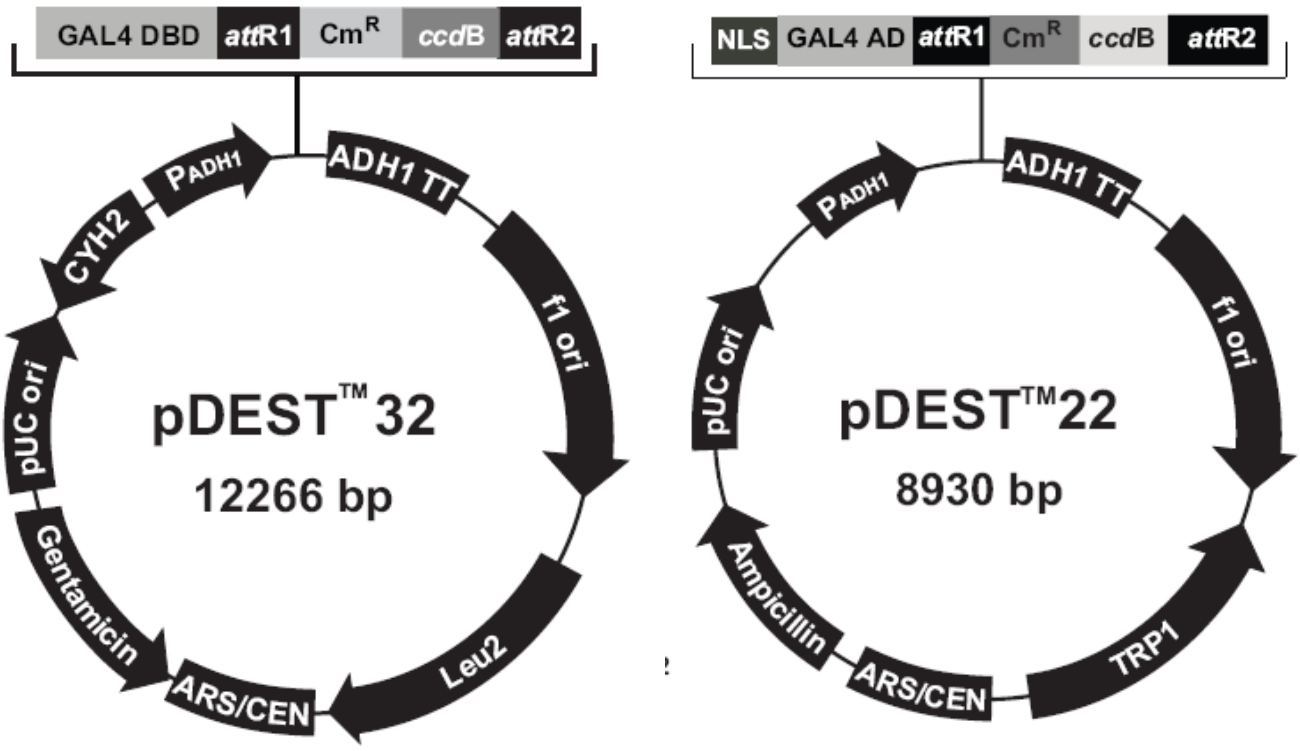


Figure 13 The map of pDEST™32 and pDEST™22 vectors

2.2 Results

The results of yeast two-hybrid assay were observed by the two main ways to check for positive interactions in yeast strain contain reporter genes: (1) Positive interactions are detected by selecting on plates lacking the auxotrophic marker, such as Histidine or Uracil. Yeast cells containing plasmids that express interacting bait and prey proteins will grow and form colonies, and (2) Positive interactions are detected by assaying for enzyme activity, such as colorimetric assays for β-galactosidase activity (Domingues *et al*. 1997). This is used to reduce false positives after selection for auxotrophs. The interpretation of yeast two-hybrid assay used in this study is shown in Table 3.

Table 3 Interpretation for yeast two-hybrid assay

	His-	***β*-Gal**	**Ura-**
Bait and Prey do not interact	**-**	**White**	**-**
Bait and Prey do interact	**+**	**Blue**	**+**

2.2.1 Interaction between Myc-RP and Myb-C05

The interaction between Myc-RP and Myb-C05 showed strong interaction between each other (Figure 14). The yeast colonies contained bait and prey plasmids of Myc-RP and Myb-C05 can growth in the selection plates without uracil or histidine and also showed the blue colonies in X-gal assay.

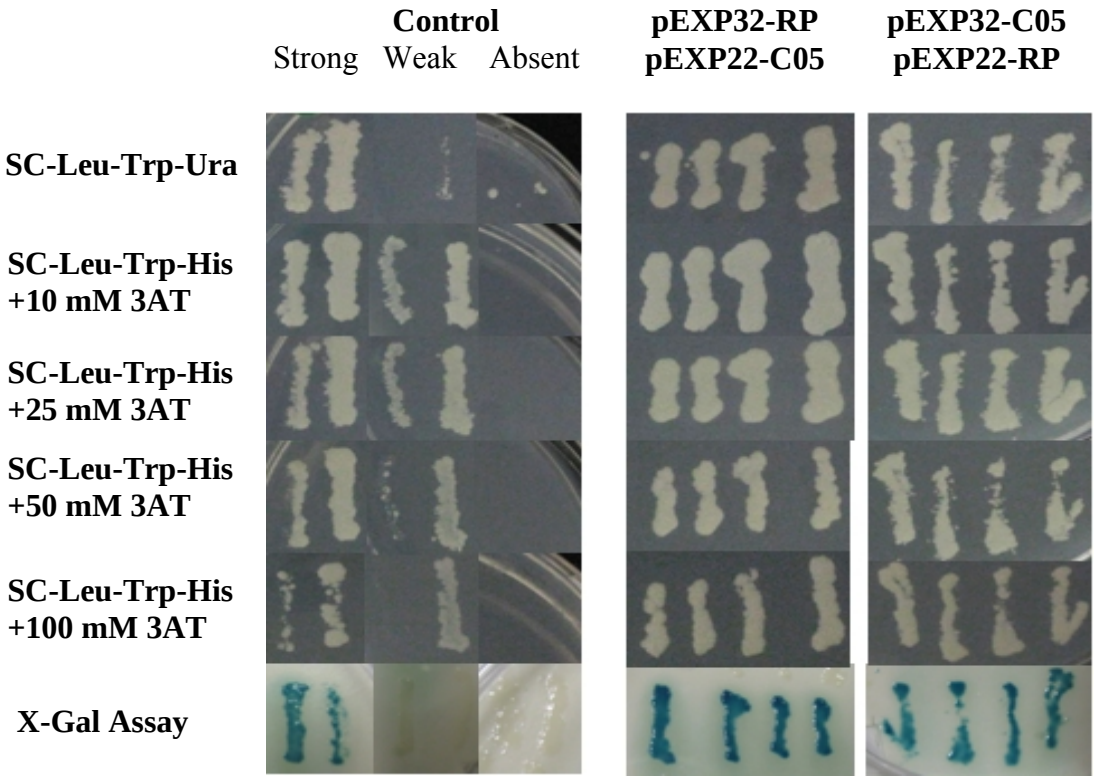


Figure 14 Interaction between Myc-RP and Myb-C05

2.2.2 Interaction between Myc-RP and Myc-RS

The interaction between Myc-RP and Myc-RS showed no interaction between each other (Figure 15).

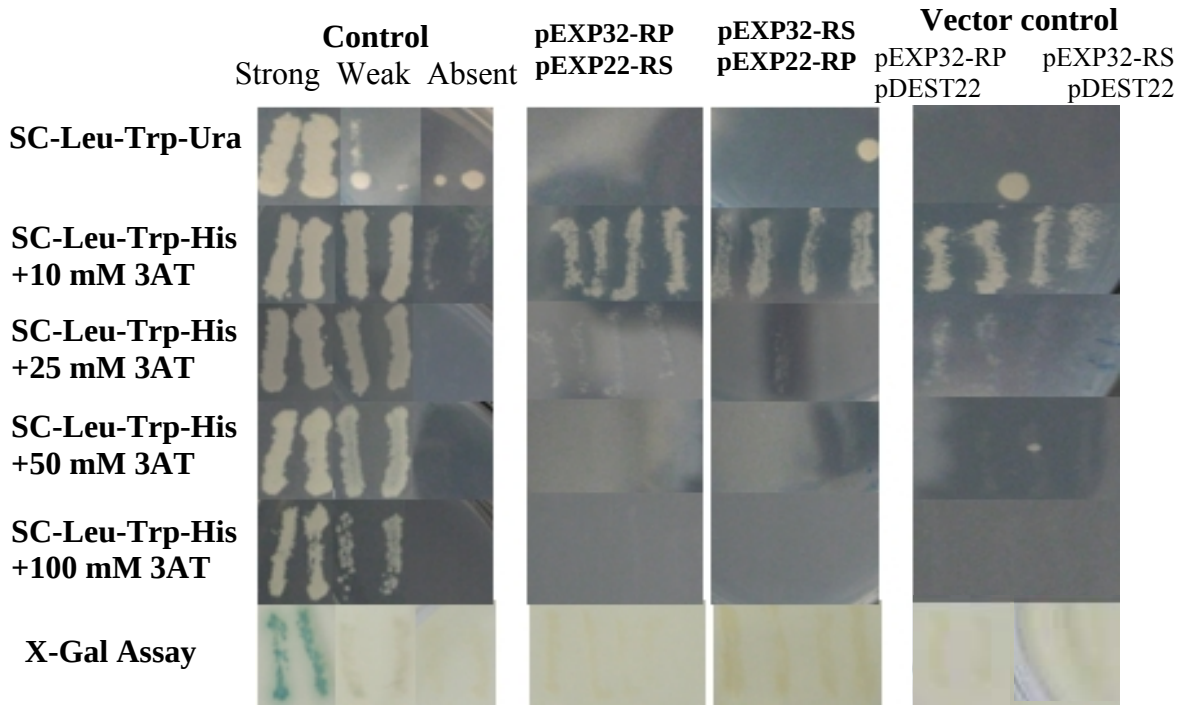


Figure 15 Interaction between Myc-RP and Myc-RS

2.2.3 Interaction between Myc-RS and Myb-C05

The interaction between the Myc-RS and Myb-C05 showed no interaction when Myb-C05 was fused to activation domain. However, showed weak interaction when Myb-C05 was fused to DNA binging domain (Figure 16).

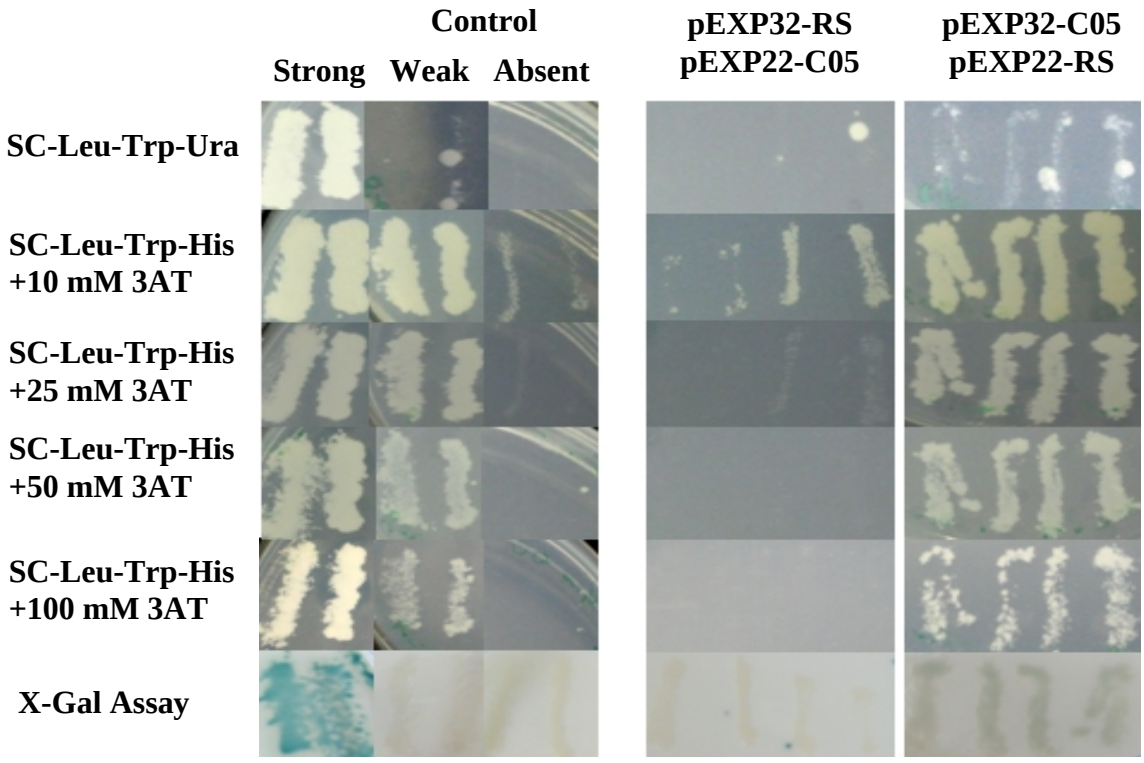


Figure 16 Interaction between Myc-RS and Myb-C05

2.2.4 Interaction between Myb-C05 and *Pf*WD

The interaction between *Pf*WD and Myb-C05 showed false positive result when compared with vector control. The results showed no interaction between *Pf*WD and Myb-C05 (Figure 17).

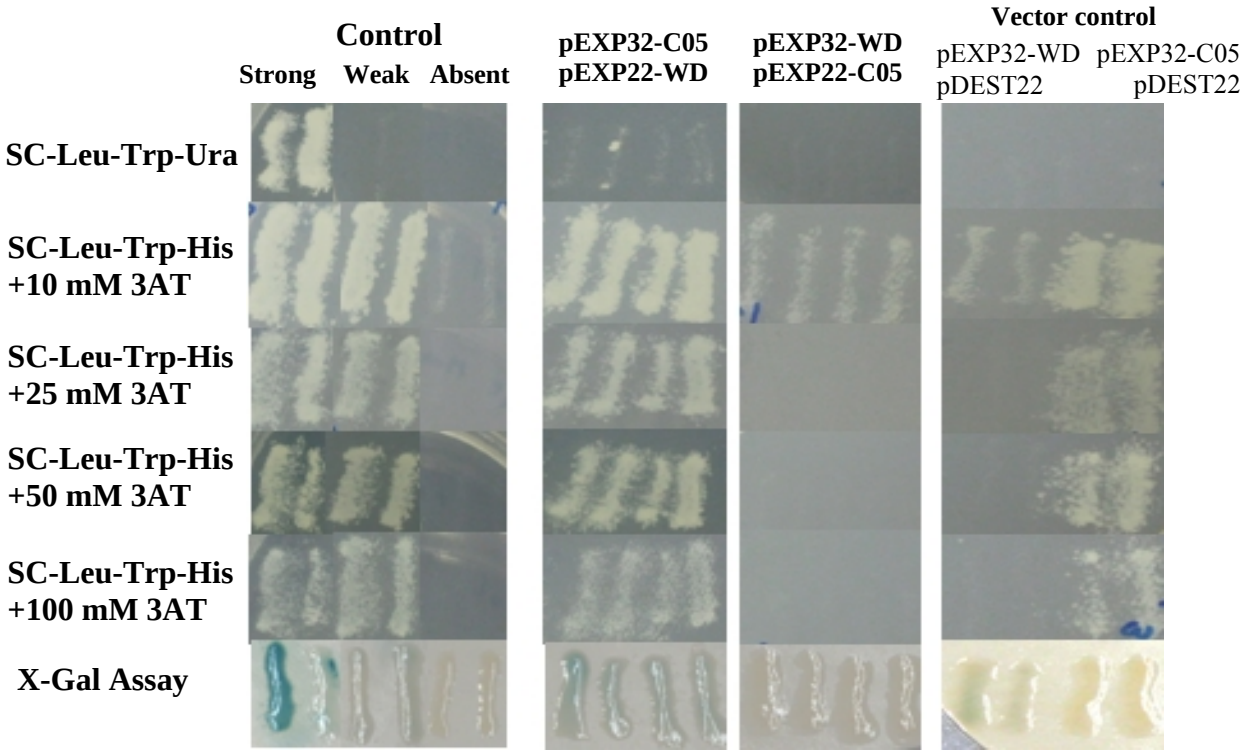


Figure 17 Interaction between Myb-C05 and *Pf*WD

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2.2.5 Interaction between Myc-RP and *Pf*WD

*Pf*WD showed strong interaction with Myc-RP when fused with activation domain and also showed weak interaction when fused with DNA binding domain (Figure 18).

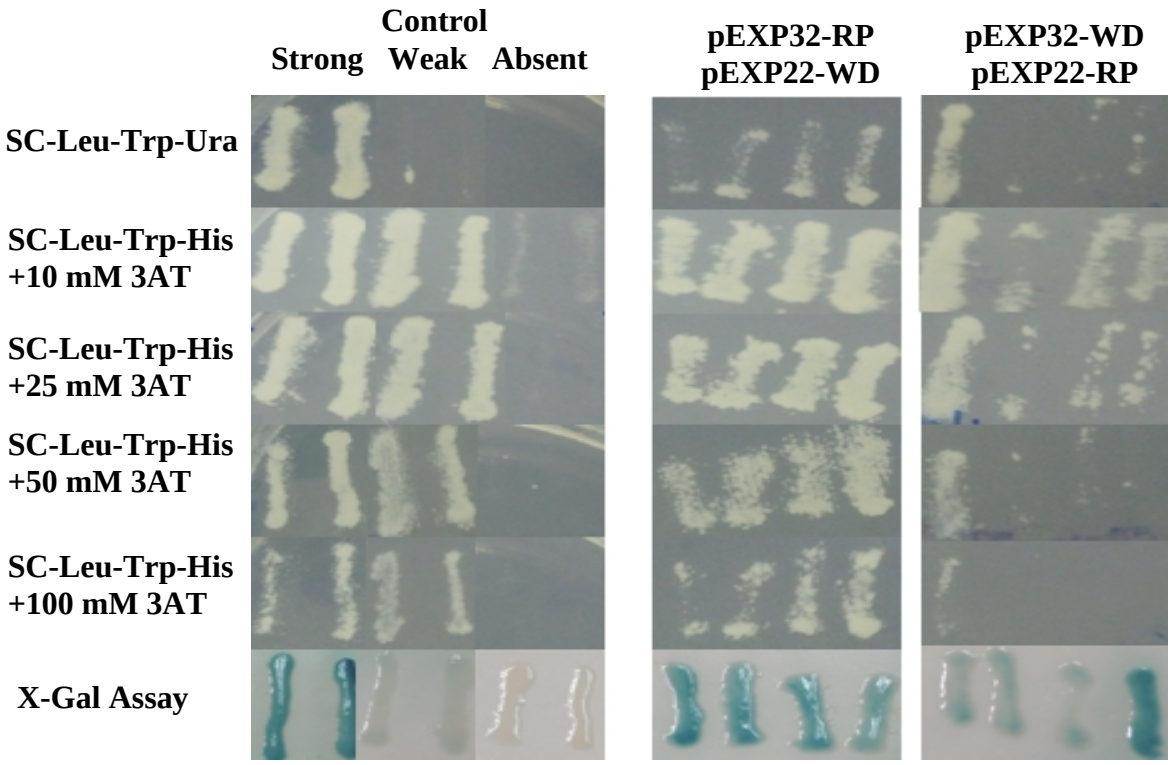

Figure 18 Interaction between Myc-RP and *Pf*WD

2.2.6 Interaction between Myc-RS and *Pf*WD

The result showed no interaction between Myc-RS and *Pf*WD (Figure 19).

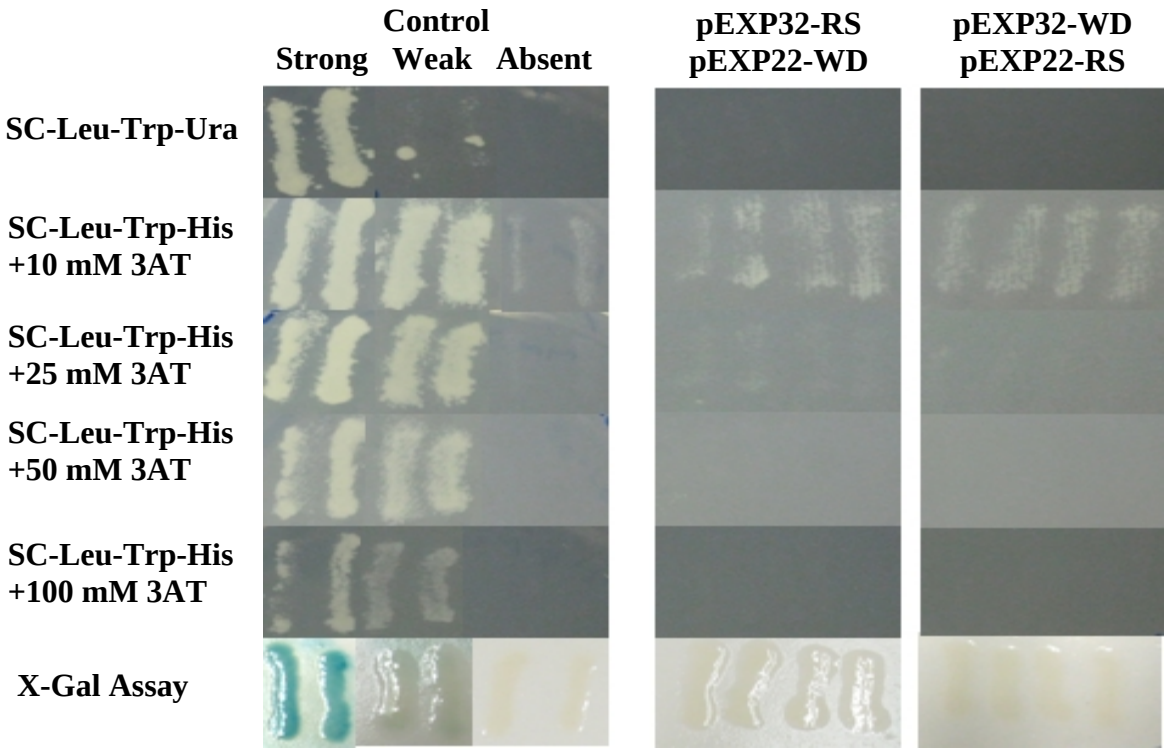

Figure 19 Interaction between Myc-RS and *Pf*WD

2.3 Discussion

From yeast two-hybrid assay results (Figure 13-Figure 18), the results can conclude as Table 4.

Table 4 Results conclusion of yeast two-hybrid assay

Interaction		Bait			
		MYC-RP	**MYC-RS**	**MYB-C05**	***Pf*WD**
Prey	**MYC-RP**		**No**	**Strong**	**Weak**
	MYC-RS	**No**		**Weak**	**No**
	MYB-C05	**Strong**	**No**		**No**
	***Pf*WD**	**Strong**	**No**	**No**	

The results suggest that Myc-RP might be involved in anthocyanin regulation. These interaction results support the role of Myc-RP in regulation of anthocynin in *P. frutescens* (Gong et al., 1999a). Myb-C05 and *Pf*WD showed no interaction to each other. Myb-C05 showed strong interaction with Myc-RP when binding to DNA binding domain, while *Pf*WD exhibited high interaction with Myc-RP when fused to activation domain. Myc-RS showed no or weak interaction to other proteins, seems that it might be not involved in anthocyanin regulation of *P. frutescens*.

From the results, the model for protein interaction in anthocyanin regulation in *P. frutescens* has been purposed as shown in Figure 20. The Myb-C05 and Myc-RS might bind to DNA binding domain. While the *Pf*WD that has been reported that localized in

cytosol (Sompornpailin *et al*. 2002), might translocate in to nucleus where the biosynthesis occurred, and interact with Myc-RP.

However, the yeast two-hybrid assay is just only screening method for protein-protein interaction. The further studies, such as the induction of enhancement of anthocyanin production in transgenic plants, should be done for clarify the role of the regulatory factors.

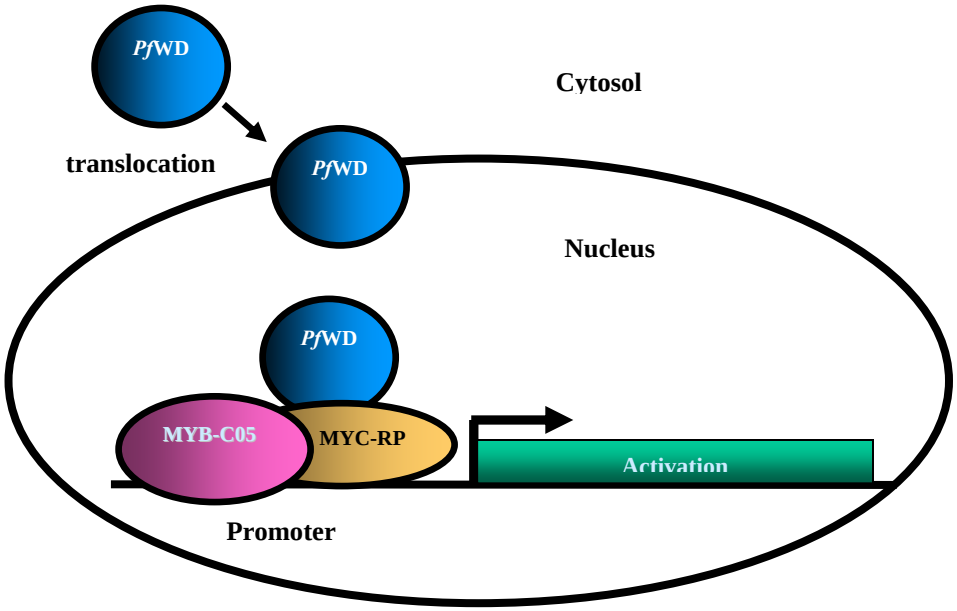

Figure 20 The suggested model for protein interaction in anthocyanin regulation in *Perilla frutescens*

GENERAL DISCUSSIONS AND CONCLUSIONS

Characterization of *GST* genes upregulated by *PAP1*

TT19 is the essential GST that require for vacuolar sequestraton of anthocyanins and proanthocyanidins (Kitamura *et al*. 2004). However, in *tt19* mutant, small amount of anthocyanins still accumulation in the mutant. Is there other GST responsible for anthocyanin accumulation in *Arabidopsis*? In *PAP1*-overexpression mutant, there are 39 genes upregulated included three GST genes, i.e. *TT19*, *AtGSTF6* and *AtGSTF5* (Tohge *et al*. 2005). *AtGSTF6* and *AtGSTF5* might be involved in anthocyanin accumulation in *Arabidopsis*. To clarify the function of the *PAP1*-upregulated GST genes, the *in vitro* GST enzymatic activity have been studied. Then, the anthocyanin accumulation levels in knockout mutants were investigated. The network analysis of co-expression using public transcriptome database were done.

The results of *in vitro* enzymatic activity using the recombinant proteins of the *PAP1*-upregulated GSTs exhibited very weak activity compared with standard GST from rat liver. The results suggest that the *PAP1*-upregulated GSTs might be not covalently binding to the substrates.

GSTs were originally termed ligandins because, in addition to their enzymic roles, they bind to large molecules leading to the suggestion that GSTs are involved in the storage and rapid transport of the compounds in plant cells. However, the precise functions of GST binding to non-substrate ligands remain unclear (Sayed *et al*. 2002).

From metabolite analysis of knockout mutants using LC-MS and the network analysis of co-expression among flanonoid related genes, the results showed among three *PAP1*-upregulated GST homologues, only *TT19* is involved in anthocyanin accumulation.

The anthocyanin regulatory factors in *Perilla frutescens*

The protein interactions between isolated regulatory genes presumably involved in anthocyanin regulation in *P. frutescens* were investigated using yeast two-hybrid assay. The results indicated the interaction between Myc-RP, Myb-C05 and *Pf*WD, which may play a role in induction of gene expression of anthocyanin biosynthesis in *P. frutescens*. The results of protein interaction of Myc-RP in yeast two-hybrid assay support the role of Myc-RP in regulation of anthocyanins in *P.frutescens* (Gong *et al*. 1999a).

However, the yeast two-hybrid assay is for screening interactions between proteins. The further studies should be done for confirm the real interaction between proteins.

CONCLUSIONS

1. Characterization of *GST* genes upregulated by *PAP1* suggested that only *TT19* (*AtGSTF12*) is involved in anthocyanin accumulation in *A. thaliana*.
2. Yeast two-hybrid experiments indicated the protein interaction of Myc-RP, Myb-C05 and *Pf*WD, which may play a role in induction of gene expression of anthocyanin biosynthesis in *Perilla frutescens*.

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MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia) plants were used as the wild-type in this study. The sterile seeds were cultured on GM-agar medium containing 1% sucrose (Valveken *et al*. 1988) in a growth chamber at 22 ºC in 16/8 h light and dark cycles for 2 weeks, and they were then transferred onto agar medium containing 10% sucrose and cultivated for 1 week in order to induce anthocyanin accumulation under high-sugar stress conditions. Rosette leaves from 3-week-old plants were harvested. For flavonoid extraction, the fresh plants were readily extracted. For DNA/RNA extraction, the plants were immediately frozen in liquid nitrogen, and stored at –80 ºC until use.

For seed sterilization, the seeds were washed in 70% EtOH and then washed in 1% sodium hypochlorite plus 0.1% of Tritron X-100 solution. The seeds in sodium hypoclorite solution were shaken for 3 min and washed with sterilized water for 5 times.

The seeds of red (Aka-jiso) and green (Ao-jiso) forms *P.frutescens* Britton var. *crispa* Thuab (Labiatae) were purchased from Sakata Seed Company (Japan). The seeds were germinated and grown at 25°C under illumination in 16 h light period in a growth chamber. After 5 weeks of germination, when the leaves had grown to a length of about 3 cm, the young leaves were used for the extraction of RNA.

Construction of GST phylogenetic tree

Amino acid sequences of fifty-three GST homologue genes in *Arabidopsis thaliana* from GenBank database, presented in the *Arabidopsis* genome initiative (AGI)

code, and the reported GST from other plants were used. Phylogenetic tree was constructed using the ClustalW program with the neighbor-joining method (Thompson e*t al*. 1994) and TreeView X.

Recombinant proteins expression in *E. coli*

Full-length *AtGSTF6* cDNA (RAFL clone no. RAFL05-16-O07) was obtained from RIKEN BioResource Center, Tsukuba, Japan. For *TT19* and *AtGSTF5*, cDNAs were isolated by RT-PCR from the leaf RNA of wild-type *Arabidopsis* by using gene-specific primers (Table 7). To express the recombinant proteins, the cDNAs of *TT19*, *AtGSTF5*, and *AtGSTF6* were introduced into the Gateway^{TM} system (Invitrogen Corp., CA, USA) following the manufacturer's instructions. The *attB* site was introduced by two steps of PCR using gene-specific primers and the *attB* adaptor primers (Table 7). Entry clones were then obtained by BP recombination reaction with pDONR221. The nucleotide sequences of the entry clones were determined to confirm the sequence. Subsequently, the cDNAs of these 3 genes were introduced into pDEST17 that contained histidine tag.

Recombinant proteins with a 6-histidine (6×His) tag at the N-terminal extension were expressed in *E. coli* BL21-AI^{TM}. Cells were grown overnight at 37 °C with shaking in 3 ml of Luria-Bertani (LB) medium containing 100 μg ml^{-1} ampicillin and then diluted 1:200 in 1 l of the same medium. Growth was monitored by measuring the turbidity at 600 nm; L-arabinose was added to a final concentration of 0.2% when the turbidity was between 0.4 and 0.6 absorption units. Incubation was continued at 20 °C for 12 h. The cells were collected by centrifugation at 10,000 × *g* for 30 min and washed with 0.9% NaCl, and the pellet was frozen at –80 °C until use. The pellet was resuspended in lysis

buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0) containing 1 mg per milliliter lysozyme and incubated on ice for 1 h. The cells were disrupted by sonication. Soluble protein extracts were obtained by centrifugation at 12,000 × *g* for 30 min. They were purified over a nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Qiagen, Maryland, USA) by using a chromatographic open column according to the manufacturer's protocol. All purification steps were carried out at 4 °C or on ice. The protein levels were analyzed using the dye-binding method (Bio-Rad, CA, USA); sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using 12% polyacrylamide gels, and Coomassie brilliant blue staining was performed.

In vitro GST enzymatic activity assay

The GST activities of the purified proteins, i.e., their ability to conjugate glutathione the universal substrate 1-chloro-2,4-dinitrobenzene (CDNB) were determined. CDNB is usually used as a model GST substrate (Edward *et al*. 2000). The absorbance at 340 nm was used to measure the amount of the conjugated product, i.e., dinitrophenol-glutathione (DNP-GS; Marrs 1996), at 25 °C in a reaction buffer containing 98 mM potassium phosphate (pH 6.5), 0.98 mM EDTA (Habig *et al.* 1974), 2 mM reduced glutathione (Wako, Osaka, Japan), and 1 mM CDNB (Wako, Osaka, Japan). GST from rat liver (Sigma-Aldrich, St. Louis, USA) was used as positive control with 2 concentrations, 0.125 μg and 0.0625 μg per reaction. The 20 μg of purified recombinant proteins were added to the reaction buffer immediately before start continuous spectrophotometric rate determination, monitoring for 30 minutes using spectrophotometer (Hitashi U-2001, Japan).

Screening of T-DNA mutants

The Salk_105779 (designated as *gstf6-1*), an *A. thaliana* (ecotype Columbia) T-DNA insertion mutant of AtGSTF6 and Salk_026398 (designated as *gstf12-1*), an insertion mutant of *TT19*, were obtained from the Salk Institute collection (Alonso et al. 2003). To confirm the T-DNA insertion and determine its position in the *gstf12-1* and *gstf6-1* lines, the genomic DNAs from the leaves were extracted using DNeasy Plant Mini Kit (Qiagen, Maryland, USA). The extracted DNAs were polymerase chain reaction (PCR)-amplified with a combination of specific primers designed for the individual lines as shown in Table 5 and LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) (http://signal.salk.edu/tdna_FAQs.html).

RT-PCR analysis for gene expression

Total RNA of wild-type and each mutants was extracted with RNeasy Plant Mini Kit (Qiagen, Maryland, USA). The cDNAs were synthesized with SyperScript™ II RNase H-reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions. The levels of *TT19* and *AtGSTF6* gene transcripts in the homozygote of the T-DNA-inserted mutants were determined by semi-quantitative reverse transcription (RT)-PCR using gene-specific primers (Table 6). The PCR condition for amplification comprised an initial denaturation step of 94 °C for 3 min; 24-30 cycles of 94 °C for 30 s, 55-60 °C for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The expression of the genes was normalized to tubulin.

Table 5 Primer design for mutant screening

Mutant	Mutant ID	LP primer	RP primer
gstf12	Salk_026398	TGAGAACCCCAAAAACGTCA	CTCATCAAGTACCCCATCGCC
gstf6	Salk_105779	CAACAACGGTTTTGTCTGTGGTC	GACCCCAAATTTGTAATTGTACCAG

Table 6 Primer design for RT-PCR analysis

Gene	AGI code	5′-primer	3′-primer
TT19	At5g17220	GGTTGTGAAACTATATGGACAGG	TCAGTGACCAGCCAGCACC
AtGSTF6	At1g02930	GAGTATTCAAGCTTGGTGGCG	CAAGACTCATTATCGAAGATTACATT
β-Tubulin	At5g62700	CCTGATAACTTCGTCTTTGG	GTGAACTCCATCTCGTCCAT

Table 7 Primer design for gene cloning and Gateway™ system

Primer	AGI code	5′-primer	3′-primer
TT19	At5g17220	AAAAAGCAGGCTCAATGGTTGTG AAACTATATGGACAGG	AGAAAGCTGGGTTCAGTGACCAGCC AGCACCATAA
AtGSTF6	At1g02930	AAAAAGCAGGCTCAATGGCAGG AATCAAAGTTTTCGG	AGAAAGCTGGGTTTAAAGAACCTTCT GAGCAGAAGGC
AtGSTF5	At1g02940	AAAAAGCAGGCTGGATGGGCAT AAACGCGAGC	AGAAAGCTGGGTTTAATTCTTCTTCT TATGGTACCAAGCC
attB adaptor		GGGGACAAGTTTGTACAAAAAA GCAGGCT	GGGGACCACTTTGTACAAGAAAGCT GGGT

Flavonoid extraction for LC/MS analysis

For flavonoid analysis, the wild-type and mutant plants were germinated in 1% sucrose GM on the same plate to minimize the differences in growth condition for two weeks. For sucrose stress anthocyanin induction, the plants were transferred to germinate in 10% sucrose for one week. The rosette leaves of the 3-week-old plants were harvested and extracted with 5 μl extraction solvent (methanol:glacial acetic acid:water = 45:5:50) per milligram fresh weight of tissue by mixer mill (MM300; Retsch Gmbl & Co. KG, Haan, Germany) at 30 Hz for 3 min. After centrifugation at 12,000 × *g*, the cell debris was discarded, and the extract was centrifuged again. The 50 μl of supernatants were used for analysis.

HPLC/PDA/ESI-MS condition

Using a high-performance liquid chromatography/photodiode array detection/electrospray ionization mass spectrometry (HPLC/PDA/ESI-MS) system comprising a Finnigan LCQ-DECA mass spectrometer (ThermoQuest, San Jose, CA, USA) and an Agilent HPLC 1100 series (Agilent Technologies, Palo Alto, CA, USA) as described previously (Jones *et al.* 2003, Yamazaki *et al.* 2003, Tohge *et al.* 2005), 50 μl of the supernatant was analyzed. HPLC was carried out on a TSK-GEL RP-18 (∅ 4.6 mm × 150 mm; TOSOH, Tokyo, Japan) at a flow rate of 0.5 ml min^{-1}. Elution gradient with solvent A [CH3CN-H2O-TFA (10:90:0.1)] and solvent B [CH3CN-H2O-TFA (90:10:0.1)] and the following elution profile (0 min 100% A, 40 min 60% A, 40.1 min 100% B, 45 min 100% B, 45.1 min 100% A, 52 min 100% A) using linear gradients in between the time points. PDA was used for detection of UV-visible absorption in the

range of 250–650 nm. Nitrogen was used as sheath gas for the positive-ion ESI-MS performed at capillary temperature and voltage of 350°C and 5.0 kV, respectively. The tube lens offset was set at 10.0 V. Full scan mass spectra were acquired from 200–1500 m/z at 2 scans sec^{-1}. Tandem MS analysis was carried out with helium gas as the collision gas. The normalized collision energy was set to 30%. Metabolites were identified based on UV visible absorption spectra and mass fragmentation by tandem MS analysis in comparison with the known compounds of laboratory stock.

Statistical data analysis

The obtained data were analyzed in term of statistic using R program (http://www.r-project.org/). Data were analyzed by one-way analysis of variance as indicated with Bonferroni *post hoc* adjustment for multiple testing.

Yeast two-hybrid plasmid construction

To perform yeast experiment, the ProQuest™ Two-Hybrid System (Invitrogen Corp., Carlsbad, CA, USA) was used. For construct yeast two-hybrid plasmid, total RNA of *P.frutescens* both red and green forms was extracted with RNeasy Plant Mini Kit (Qiagen, Maryland, USA), and cDNA was synthesized with SuperScript™ II RNase H-reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions. The gene specific primers, as shown in Table 8, were used to obtain the full-length of each gene. The full-length cDNAs of *Myc-RP*, *Myc-RS*, *Myb-C05*, and *PfWD* were introduced into the Gateway™ system (Invitrogen Corp., CA, USA) following the manufacturer's protocol. The *attB* site was introduced by two steps

of PCR using gene-specific primers (Table 8) and the *attB* adapter primers (Table 7). Entry clones were then obtained by BP recombination reaction with pDONR221. The nucleotide sequences of the entry clones were determined to confirm the sequence. Then, the entry clones of these 4 genes were introduced into pDEST™32 (bait vector) and pDEST™22 (prey vector) using LR recombination reaction. The yeast expression vector pDEST™32 and pDEST™22 is for generation of GAL4 DNA Binding Domain (GAL4 DBD) and GAL4 Activation Domain (GAL4 AD) fusion protein, respectively. The bait and prey plasmids were transformed to *E. coli* DH5α™. The primers used for confirm the insertions were shown in Table 8.

The yeast control plasmids were also constructed. Using the control vectors provided with the kit, four two-hybrid control plasmids based on the interaction of Krev1 (a.k.a. Rap1A; a member of the Ras family of GTP binding proteins) with RalGDS (the Ral guanine nucleotide dissociator stimulator protein (Herrmann *et al*. 1996, Serebriiskii *et al.* 1999). The RalGDS mutants RalGDS-m1 and RalGDS-m2 affect the interaction with Krev1 and were generated using the SureFrame™ Allele Library Construction Kit. The positive and negative control plasmids were constructed. The properties of these plasmids are summarized in Table 9.

Yeast transformation

The yeast (*Saccharomyces cerevisiae*) strain MaV203 is genetically modified and contained three GAL4 inducuble reporter genes stably integrated into the genome. The competent cell of the yeast was prepared by using *S.c*. EasyComp™ Kit (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's manual. The bait and prey plasmids,

contained gene of interest or the control vectors, were transformed, following the protocol provided with the kit, to yeast competent cells and plated on a selection plate, SC drop out medium without leucine and tryptophan (SC-Lue-Trp). The plates were incubated at 28°C for 2-4 days. The growth of yeast colonies were used as indicator for transformation success.

Characterization of transformants

The yeast MaV203 cells that contain bait and prey proteins that strongly interact will induce all three reporter genes present in this system (*HIS3*, *URA3*, *lacZ*). To test specific interaction, the plates described in Table 10 were used for assay. For characterize the transformants, the master plates were generated and incubated at 30°C for 18 h, then the colonies from the master plates were used for testing the reporter genes. The replica plating and cleaning technique was used for reduce background. The selection plates were replica plated and incubated for 24 h at 30°C. After that, the replica cleans were generated and the results were determined after incubated at 30°C for 2 days.

X-gal assay

The Xgal substrate is commonly used since its β-galactosidase hydrolysis product dimerizes and forms a blue precipitate. The YPAD containing a nitrocellulose were replica plated from master plates and incubated at 30°C for 24 h. The X-gal solution was prepared following the protocol. The membranes were removed from the surface of YPAD plates and completely immersed in liquid nitrogen for 30 s. The membranes were placed on the filter papers soaked with X-gal solution and incubated at 37°C for 24 h.

The appearance of blue color was monitored over a 24-h period and scored the final results at 24 h.

Table 8 Primer design for gene cloning and yeast two-hybrid system

Primer	5′-primer	3′-primer
MYC-RP	AAAAAGCAGGCTTTATGGCTACTGCAAATCAAAAGGGTG	AGAAAGCTGGGTTCAACCCACTTTTTGATTATTCTCTGAA
MYC-RS	AAAAAGCAGGCTTTATGAGGCTTGAGGATGACTCTCAGC	AGAAAGCTGGGTTTACCCGAGAGATAGTTGATGGGGAG
MYB-C05	AAAAAGCAGGCTACATGGCTGCTGATGAGGAGCCCAAC	AGAAAGCTGGGTTTCATGCATCTTCCTCGAGTTCAA
PfWD	AAAAAGCAGGCTCGATGGAAAAT TCGACCCAGGAATCCC	AGAAAGCTGGGTCTCACACTTTCAGCATTTGCATCTTG
Bait	AACCGAAGTGCGCCAAGTGTCTG	AGCCGACAACCTTGATTGGAGAC
Prey	TATAACGCGTTTGGAATCACT	AGCCGACAACCTTGATTGGAGAC

Table 9 The control plasmids used in yeast two-hybrid assay

Control	LEU2 Plasmid	TRP1 Plasmid	Purpose
1	None	None	Negative transformation control
2	pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
3	pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
4	pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
5	pDEST™32	pDEST™22	Negative activation control
6	Bait plasmid	pDEST™22	Negative activation control, baseline

Table 10 The mediums used for characterization if transformants

Test	*HIS3* induction	*URA3* induction	β-Galactosidase induction
Assay	His auxotrophy	Uracil auxotrophy	X-gal assay
Plates used	SC-Leu-Trp-His+3AT	SC-Leu-Trp-Ura	YPAD
Concentrations	10 mM 3AT 25 mM 3AT 50 mM 3AT 100 mM 3AT	No Uracil	Not applicable

REFERENCES

Abrahams S, Tanner GJ, Larkin PJ, Ashton AR (2002) Identification and biochemical characterization of mutants in the proanthocyanidin pathway in *Arabidopsis*. *Plant Physiol* **130**: 561–576

Alfenito MR, Souer E, Godman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanins sequestration in the vacuole by widely divergent glutathione *S*-transferases. *Plant Cell* **10**: 1135–1149

Alonso JM, Stepanova AN, Leisse TJ *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–657

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L (2004) *TT2*, *TT8*, and *TTG1* synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant J*. **39**: 366–380

Bogs J, Downey MO, Harvey JS, Ashton AR, Tanner GJ, Robinson SP (2005) Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol* **139**: 652–663

Borevitz J, Xia Y, Blount JW, Dixon RA, Lamb C (2001) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* **12**: 2383–2393

Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev*. **56**: 317-333

Buetler TM, Eaton DL (1992) Glutathione *S*-transferases: amino acid sequence comparison, classification and phylogenetic relationship. *Environmental Carcinogen and Ecotoxicology Reviews* **C10**, 181–203

Carey CC, Strahle JT, Selinger DA, Chandler V (2004) Mutations in the *pale aleurone color1* regulatory gene of the *Zea mays* anthocyanin pathway have distinct phenotype relative to the functionally similar *Transparent Testa Glabra1* gene in *Arabidopsis thaliana*. *Plant Cell* **16**: 450–464

Chandler VL, Radicella JP, Robbins TP, Chen J, Turks D (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of b utilizing r genomic sequences. *Plant Cell* **1**:1175–1183

Cho HY, Kong KH (2003) Molecular cloning, expression, and characterization of a phi-type glutathione *S*-transferase from *Oryza sativa. Pest. Biochem. Physiol.* **83**: 29–36

Cone KC, Burr FA, Benjamin B (1986) Molecular analysis of the maize anthocyanin regulatory locus *c1*. *Proc. Natl Acad. Sci. USA* **83**: 9631–9635

Davies KM, Schwinn KE (2003) Transcriptional regulation of secondary metabolism. *Functional Plant Biology* **30**: 913-925

Dixon DP, Davies BG, Edwards E (2002b) Functional divergence in the glutathione transferase superfamily in plants. *J Biol Chem* **277**: 30859–30869

Dixon DP, Lapthorn A, Edwards R (2002a) Plant glutathione transferases. *Genome Biol* **3**: 3004.1–3004.10

Dixon RA, Xie DY, Sharma SB (2005) Proanthocyanidins—a final frontier in flavonoid research? *New Phytol*. **165**: 9-28

Domingues L, Teiseira J, Lima N (1997) Rapid and sensitive detection of *β*-galactosidase-producing yeasts by using microtiter plate assay. *Biotechnology Techniques* **11**: pp. 399–402

Droog FJN, Hooykaas PJJ, van der Zaal BJ (1995) 2,4 Dichlorophenoxyacetic acid and related chlorinated compounds inhibit two auxin-regulated type-III tobacco glutathione *S*-transferases. *Plant Physiol.* **107**: 1139–1146

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci* **5**: 193–198

Fields S, Song O (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340**: 245 – 246

Frova C (2003) The plant glutathione transferase gene family: genomic structure, functions, expression and evolution. *Physiol. Plant* **119**: 469–479

Goff SA, Cone KC, Chandler VL (1992) Functional analysis of the transcription activator encoded by the maize *B*-gene: Evidence for a direct functional interaction between two classes of regulatory proteins. *Genes Dev*. **6**, 864–875

Gong Z, Yamazaki M, Sugiyama M, Tanaka Y, Saito K (1997) Cloning and molecular analysis of structural genes involved in anthocyanin biosynthesis and expressed in a forma specific manner in *Perilla frutescens*. *Plant Mol. Biol.* **35**: 915–927

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant J.* **53**: 814–827

Goodman CD, Casati P, Walbot V (2004) A multidrug resistance associated protein involved in anthocyanin transport in *Zea mays*. *Plant Cell* **16**: 1812–1826

Grotewold E (2004) The challenges of moving chemicals within and out of cells: insights into the transport of plant natural products. *Planta* **219**: 906-909

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize *C1* that specify the interaction with the bHLH cofactor *R*. *Proc. Natl. Acad. Sci. USA* **97**: 13579-13584

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferase: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**: 7130-7139

Harborne J, Williams C (2000) Advances in flavonoid research since 1992. *Phytochemistry* **55**: 481-504

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci USA* **98**: 4569–4574

Jin H, Martin C (1999) Multifunctionality and diversity within the plant *MYB*-gene family. *Plant Mol Biol* **41**:577-585

Jones P, Messner B, Nakajima J, Schäffner AR, Saito K (2003) UGT73C6 and UTG78D1, glycosyltransferases involved in flavonol glycoside biosynthesis in *Arabidopsis thaliana*. *J. Biol. Chem.* **45**: 43910-43918

Jung M, Chung H, Choi J (2001) Antioxidant activity of roasted defatted *Perilla* seed. *Nat. Prod. Sci.* **7**: 72-75

Kitamura S, Shikazono N, and Tanaka A (2004) *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *Plant J* **37**: 104–114

Kitamura S (2006) Transport of flavonoids. In The Science of Flavonoids Edited by: Grotewold E. Springer: 123-146

Koes R, Verweij CW, Quattrocchio F (2005). Flavonoids: A colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* **5**: 236–242

Makino T, Furata Y, Wakushima H, Fujii H, Saito K, Kano Y (2003) Anti-allergic effect of *Perilla frutescen*s and its active constituents. *Phytother. Res.* **17**: 240-243

Marrs KA (1996) The functions and regulation of glutathione *S*-transferases in plants. *Annu Rev Plant Physiol Plant Mol Biol* **47**: 127–158

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

Martin C, Paz-Ares J (1997) MYB transcription factors in plants. *Trends Genet.* **13**: 67–73

McGonigle B, Keeler SJ, Lan SMC, Koeppe MK, O'Keefe DP (2000) A genomics approach to the comprehensive analysis of the glutathione *S*-transferase gene family in soybean and maize. *Plant Physiol* **124**:1105–1120

McMahon LR, McAllister TA, Berg BP, Majak W, Acharya SN, Popp JD, Coulman BE, Wang Y, Cheng KJ (2000) A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Can J Plant Sci* **80**: 469–485

Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. *Trends Plant Sci* 3: 212–217

Morita Y, Saitoh M, Hoshino A, Nitasaka E, Iida S (2006) Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of c and ca mutations conferring white flowers in the Japanese morning glory. *Plant Cell Physiol.* **47**: 457–470

Mueller LA, Goodman CD, Silady RA, Walbot V (2000) *AN9*, a petunia glutathione *S*-transferase required for antocyanin sequestration, is a flavonoid-binding protein. *Plant Physiol.* **123**: 1561–1570

Murre C, McCaw PS, Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* **56**: 777-783

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The Arabidopsis *TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell* **13**: 2099–2114.

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcription factors. *EMBO J.* **6**: 3553– 3558

Peer WA, Brown DE, Tague BW, Muday GK, Taiz L, Murphy AS (2001) Flavonoid accumulation patterns of *transparent testa* mutants of *Arabidopsis*. *Plant Physiol.* **126**:536-48

Poustka F, Irani NG, Feller A, Lu Y, Pourcel L, Frame K, Grotewald E (2007). A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-

vacuole protein sorting route in *Arabidopsis* and contributes to the formation of vacuolar inclusions. *Plant Physiol*. **145**: 1323–1335

Quackenbush J (2001) Computational analysis of microarray data. *Nat Rev Genet.* **2**: 418-427

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

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J, Koes R (1999) Molecular analysis of the *Anthocyanin2* gene of Petunia and its role in the evolution of flower color. *Plant Cell* **11**: 1433–1444

Ramsay NA, Glover BJ (2005) MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends in Plant Science* **10**: 63-70

Ricarda Da Silva JM, Darmon N, Fernandez Y (1991) Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. *J. Agri. Food Chem.* **39**:1549-1552

Riechmann JL, Heard J, Martin G, Reuber L, -Z. C, Jiang, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, -L. Yu G (2000) Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes. *Science* **290**: 2105-2110

Santos-Buelga C, Scalbert A (2000) Proantocyanidins and tannin-like compounds: nature, occurrence dietary intake and effects on nutrition and health. *J. Sci. Food Agri.* **80**:1094-1117

Sayed Y, Hornby JA, Lopez M, Dirr H (2002) Thermodynamics of the ligandin function of human class *Alpha* glutathione transferase *A1-1*: energetics of organic anion ligand binding. *Biochem. J.* **363**: 341–346

Schwinn K, Venail J., Shang Y, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davies K, Martin C (2006) A small family of *MYB*-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* **18**: 831–851

Smith TF, Gaitatzes C, Saena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. *Trends Biochem Sci* **24**: 181-185

Sompornpailin K, Makita Y, Yamazaki M, Saito K (2002) A WD-repeat-containing putative regulatory protein in anthocyanin biosynthesis in *Perilla frutescens*. *Plant Mol. Biol.* **50**: 485–495

Spelt C, Quattrocchio F, Mol JN, Koes R (2000) *Anthocyanin1* of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. *Plant Cell* **12**: 1619–1632

Springob K, Nakajima J, Yamazaki M, Saito K (2003) Recent advances in the biosynthesis and accumulation of anthocyanins. *Nat. Prod. Rep.* **20**: 288-303

Stapleton A, Walbot V (1994) Flavonoids protect maize DNA from UV damage. *Plant Physiology* **105**: 881-889

Stuart JM, Segal E, Koller D, Kim SK (2003) A gene coexpression network for global discovery of conserved genetic modules. *Science* **302**: 249–255

Takano H, Osakabe N, Sanbongi C, Yanagisawa R, Inoue K, Yasuda A, Natsume M, Baba S, Ichiishi E, Yoshikawa T (2004) Extract of *Perilla frutescens* enriched

for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans. *Exp. Biol. Med.* **229**: 247-254

Tanaka Y, Sasaki N, Ohmiya A (2008) Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* **54**:733-49

Taylor LP, Briggs WR (1990) Genetic regulation and photocontrol of anthocyanin accumulation in maize seedlings. *Plant Cell* **2**: 115–127

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673-4680

Tohge T, Nishiyama Y, Hirai MY, Yano M, Nakajima J, Awazuhara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M, Noji M, Yamazaki M, Saito K (2005) Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. *Plant J* **42**: 218–235

Ueda H, Yamazaki C, Yamazaki M (2002) Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. *Biol. Pharm. Bull.* **25**: 1197-1202

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium*-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. *Proc. Natl Acad. Sci. USA* **85**: 5536–5540

Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JP (2008) The neuroprotective potential of flavonoids: a multiplicity of effects. *Genes Nutr.* **3**: 115–126

de Vetten N, Quattrocchio F, Mol J, Koes R (1997) The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. *Genes Dev.* **11**: 1422–1434

Wagner U, Edwards R, Dixon DP, Mauch F (2002) Probing the diversity of the Arabidopsis glutathione *S*-transferase gene family. *Plant Mol Biol* **49**: 515–532

Walhout AJ, Boulton SJ, Vidal M (2000) Yeast two-hybrid systems and protein interaction mapping projects for yeast and worm. *Yeast*. **17**: 88-94

Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL., Esch JJ, Marks MD, Gray JC (1999) The *Transparent Testa Glabra1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* 11: 1337–1350

Wang H, Cao G, Prior RL (1997) Oxygen radical absorbing capacity of anthocyanins. *J Agric Food Chem.* **45**: 304–309

Winkel-Shirley B (1999) Evidence of enzyme complexes in the phenylpropanoid and flavonoid pathways. *Physiol Plant* **107**: 142-149

Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology and biotechnology. *Plant Physiol* **126**: 485-493

Winkel-Shirley B (2004) Metabolic channeling in plants. *Annu Rev Plant Biol* **55**: 85–107

Yamasaki K, Nakano M, Kawahata T, Mori H, Otake T, Ueba N, Oishi I, Inami R, Yamane M,Nakamura M, Murata H, Nakanishi T (1998) Anti-HIV-1 activity of herbs in Labiatae. *Biol. Pharm. Bull.* **21**: 829-833

Yamazaki M, Nakajima J, Yamanashi M, Sugiyama M, Makita Y, Springgob K, Awazuhara M, Saito K (2003) Metabolomics and differential gene expression in anthocyanin chemo-varietal forms of *Perilla frutescens*. *Phytochemistry* **62**: 987-995

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all *TTG1*-dependent pathways of *Arabidopsis*. *Development* **130**: 4859–4869

Zhang P, Chopra S, Peterson T (2000) A segmental gene duplication generated differentially expressed *myb*-homologous genes in maize. *Plant Cell* **12**: 2311–2322

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. *Plant J*. **40**: 22–34

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THESIS COMMITTEE

This thesis was submitted to the Graduate School of Pharmaceutical Sciences, Chiba University, Japan, in fulfillment of the requirements for the degree of Doctorate of Philosophy (Ph. D.) and had been examined by the following thesis committee authorized by the Graduate School of Pharmaceutical Sciences, Chiba University.

Chairman: Naoto Yamaguchi, Ph.D. (Pharm. Sci.), Professor of the Graduate School of Pharmaceutical Sciences, Chiba University.

Members: Masami Ishibashi, Ph.D. (Sci.), Professor of the Graduate School of Pharmaceutical Sciences, Chiba University.

Members: Osamu Ohara, Ph.D., Professor of Kazusa DNA Research Institute, Chiba University.

研 究 業 績 発 表 会 要 旨

Functional analysis of genes involved in anthocyanin accumulation in *Arabidopsis thaliana*

（シロイヌナズナにおけるアントシアニン蓄積に関する遺伝子の機能解析）

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INTRODUCTION

Flavonoid biosynthesis is the one of the most intensively studied secondary metabolisms. Anthocyanins and proanthocyanidins are a large subclass of flavonoid pigments that provide important functions in plants. Anthocyanins are secondary compounds unique to plants and responsible for brightly colored pigments produced in flowers, fruits, and leaves that have main function to attract pollinators and also protect the tissues from UV damage. Interest in anthocyanins increased due to their health-promoting activities. Anthocyanins possess considerable antioxidant properties and are therefore believed to reduce the risk of coronary heart disease and cancer. The low toxicity of these compounds makes them valuable nutraceuticals. Proanthocyanidins are the secondary metabolites that have major role to provide protection against microbial pathogens, insect pests and larger herbivores. Proanthocyanidins present in the fruits, bark, leaves and seed of many plants.

In plant cells, both anthocyanins and proanthocyanidins are normally localized in the vacuole; however, the enzymatic steps of their biosynthesis occur in the cytoplasm. So far, it is known that the homologue proteins of glutathione *S*-transferase (GST) plays an important role in the transport and accumulation of anthocyanins and proanthocyanidins. In a model plant *Arabidopsis thaliana*, *TRANSPARENT TESTA 19* (*TT19)*, encoding a GST homologue, was investigated to be required for the accumulation of anthocyanins and proanthocyanidins. In previous study on *Arabidopsis thaliana* overexpressing the *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*) gene encoding an MYB transcription factor (MYB75), the genes encoding GST-like proteins, *AtGSTF5* and *AtGSTF6,* were up-regulated along with *TT19*. To clarify the role of these GST homologous genes, the present research investigated the enzymatic activities of recombinant proteins and the metabolite change in knockout mutants of *TT19* and *AtGSTF6* using LC-MS (**PART I**).

Perilla frutescens var. *crispa,* or “Shiso” in Japanese, is a medicinal plant used in Japan and China. This species is a useful model to study the regulatory mechanism of anthocyanin biosynthesis, because it exists in two chemo-varietal forms, namely the green-form “Ao-shiso” and the red-form “Aka-shiso”. The red-form accumulates large amounts of anthocyanins in leaves, stems and flowers, whereas the green-form contains only traces of anthocyanins. From previous study, the structural genes encoding enzymes in anthocyanin biosynthesis in *P. frutescens* are coordinately regulated by possible regulatory genes; however, the detailed regulatory system is still unclear in *P. frutescens*. In all higher plants studied to date, the anthocyanin pigment pathway is regulated by a suite of transcription factors that include Myb, bHLH and WD-repeat proteins. In this study, the protein interaction of regulatory factor candidates from *P. frutescens* was investigated using yeast two-hybrid system for understanding the anthocyanin regulatory system (**PART II**).

RESULTS AND DISCUSSION

PART I. Characterization of *PAP1*-upregulated glutathione *S*-transferase genes in *Arabidopsis thaliana*

1.The enzymatic activity of GST homologue proteins

The recombinant proteins of *At*GSTF5, *At*GSTF6 and TT19 were expressed in *Escherichia coli*. The soluble protein extracts were purified and used for *in vitro* GST enzymatic activity assay. The GST activities of purified proteins were determined by the ability of recombinant proteins to conjugate glutathione to the model substrate, 1-chloro-2,4-dinitrobenzene (CDNB). All recombinant proteins showed GST activities, indicating that all genes encoded glutathione *S*-transferase enzymes. However, the activities were very low compared with the authentic GST from mammals.

2. Metabolic change in the knockout mutants of GST homologue genes

The T-DNA insertion mutants of *AtGSTF6* and *TT19* were obtained from the Salk institute collection, and then the T-DNA insertion and knockout of gene expression were confirmed (Figure 1). The rosette leave extracts from wild type (WT) and mutant plants were analyzed for the accumulation of flavonoids using LC-MS. The anthocyanin accumulation in *tt19* knockout mutant, decreased significantly compared with that in wild-type plants; however, no changes in the anthocyanin pattern could be detected. In contrast, both anthocyanin level and pattern of *gstf6* knockout mutant showed no significant changes. For flavonoid accumulation and pattern, both *tt9* and gstf6 knockout mutants showed no significant changes (Figure 2). These results suggest that *TT19* is involved in anthocyanin accumulation, causing the change in anthocyanin accumulation, whilst *AtGSTF6* might be not involved in anthocyanin biosynthesis.

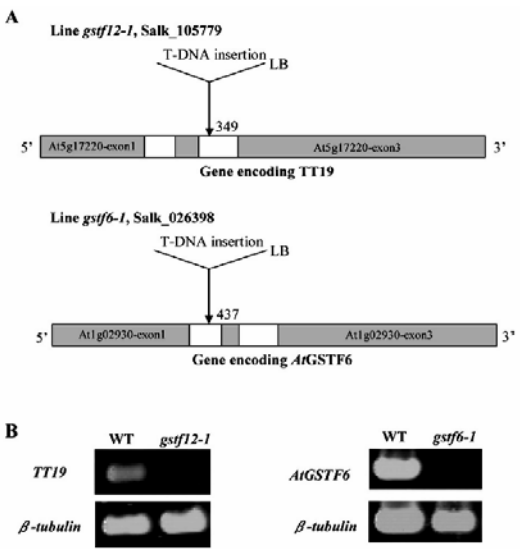

Figure 1 A. Schematic structure of the T-DNA-inserted lines of *TT19* (At5g17220) (line *gstf12-1*, Salk_105779) and *AtGSTF6* (At1g02930) (line *gstf6-1*, Salk_026398). **B.** Expression of the *TT19* and *AtGSTF6* genes in mutant plants compared with that in wild-type Col-0 (WT) plant.

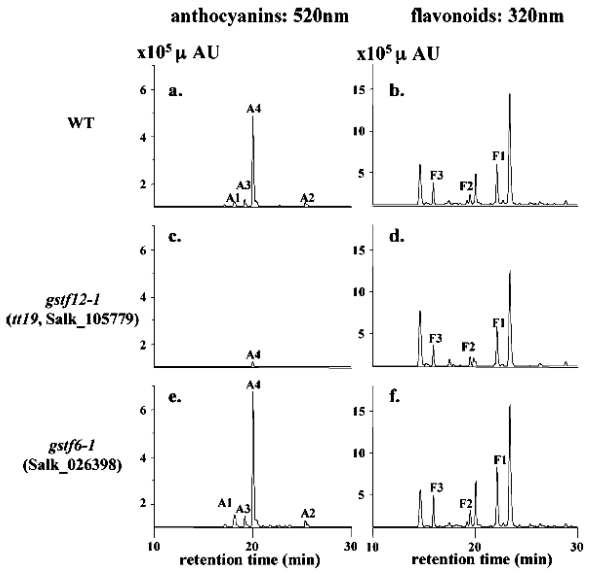

Figure 2 HPLC/PDA chromatograms of rosette leaf extract from wild-type (WT) *Arabidopsis*, the *tt19* knockout mutant (*gstf12-1*), and the *AtGSTF6* knockout mutant (*gstf6-1*). (a, c, e) Absorbance at 520 nm for detection of anthocyanins. (b, d, f) Absorbance at 320 nm for detection of flavonoids.

3. Network analysis of co-expression of flavonoid related transcriptome

The networks co-expression analysis of transcriptome is a tool for determination of the relation between genes. The clusters of genes with related functions often exhibit similar expression patterns that are co-expressed in DNA microarray experiments in various conditions. The results of co-expression network analysis using public transcriptome data corresponded to the proposition of the predominant role of *TT19* in anthocyanin accumulation (Figure 3).

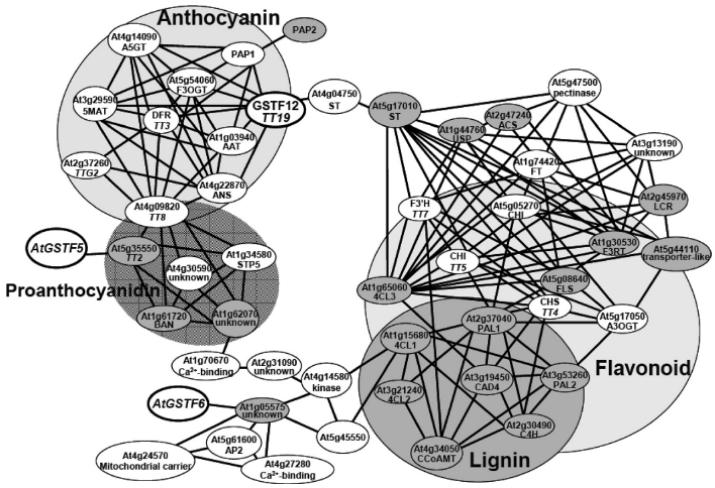

Figure 3 Co-expression networks of *PAP1*-upregulated genes with whole genes.

CONCLUSIONS

This study indicates that, among three *PAP1*-upregulated GST genes, only *TT19* is involved in anthocyanin accumulation, causing the change in anthocyanin accumulation, whist *AtGSTF6* exhibiting *in vitro* GST activity is not involved in anthocyanin biosynthesis.

PART II. Regulatory mechanism of anthocyanin biosynthesis in *Perilla frutescens* approached by yeast two-hybrid system

The four regulatory gene candidates (*MYC-RP*, *MYC-RS*, *MYB-C05* and *PfWD*) previously isolated from *P. frutescens* were fused to the DNA-binding domain, called as 'Bait', and the activation domain, called as 'Prey', for use in yeast two-hybrid studies. The constructs were transformed to express in MaV203 yeast strain. Strain MaV203 carries *URA3*, *HIS3* and *lacZ* reporter genes. Transcriptional activation of these reporter genes was analyzed (i) by growth on uracil-deficient selective dropout plates, (ii) by growth on histidine-deficient selective dropout plates and (iii) by the formation of blue colonies on plates containing X-Gal. The results showed MYC-RP has strong interaction with MYB-C05 and *Pf*WD protein in the yeast system. These results support the role of MYC-RP in regulation of anthocyanin in *P. frutescens*. MYB-C05 and *Pf*WD might be also involved in regulation. The model for anthocyanin regulation in *P. frutescens* can be suggested as Figure 4.

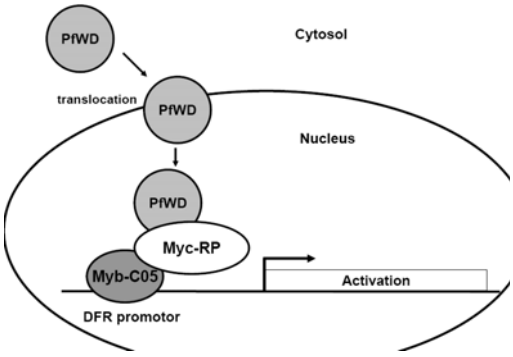

Figure 4 Suggested model for anthocyanin regulation in *Perilla frutescens*.

PUBLICATION

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