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Gene Note

Characterization of *PAP1*-upregulated Glutathione *S*-transferase genes in *Arabidopsis thaliana*

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Abstract Glutathione *S*-transferase (GST) plays an important role in the transport and accumulation of anthocyanin and proanthocyanidin in plants. In our previous study on *Arabidopsis thaliana* overexpressing the *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*) gene encoding an MYB transcription factor, the *AtGSTF5* and *AtGSTF6* genes encoding GST-like protein were up-regulated along with *TRANSPARENT TESTA 19* (*TT19*), which is required for the accumulation of anthocyanin and proanthocyanidin. The proteins encoded by these 3 genes showed very weak GST activities as detected by using recombinant proteins expressed in *Escherichia coli.* The anthocyanin levels were severely decreased in the *tt19* mutant but not in the *Atgstf6* mutant, suggesting that *TT19* is almost exclusively involved in anthocyanin accumulation. The results of co-expression network analysis using public transcriptome data corresponded to the proposition of the predominant role of *TT19* in anthocyanin accumulation.

Key words: Anthocyanin, *Arabidopsis thaliana*, glutathione *S*-transferase, *TT19.*

Flavonoid biosynthesis is the one of the most intensively studied secondary metabolite pathways. Anthocyanins and proanthocyanidins (or condensed tannins) are a large subclass of flavonoid pigments that perform important functions in plants (Winkel-Shirley 2001). Anthocyanins are brightly colored pigments produced in flowers and fruits whose main function is to attract pollinators and seed dispersers (Mol et al. 1998). Proanthocyanidins are secondary metabolites whose major role is to provide protection against microbial pathogens, insect pests, and herbivores. Proanthocyanidins are present in the fruits, bark, leaves, and seeds of many plants (Dixon et al. 2005). Both 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). Glutathione *S*-transferase (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*

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione (reduced); GST, glutathione *S*-transferase
This article can be found at http://www.jspcmb.jp/

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, we examined the enzymatic activities of recombinant proteins and the metabolite changes in knockout mutants of these *GST*-like genes.

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). The plants were germinated on agar medium containing 1% sucrose (Valvekens et al. 1988) at 22°C 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, immediately frozen in liquid nitrogen, and stored at −80°C until use.

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 (*gstf12-1*: left, 5′-TGAGAACCCCAAAAACGTCA-3′ and right, 5′-CTCATCAAGTACCCCATCGCC-3′; *gstf6-1*: left, 5′-CAACAACGGTTTTGTCTGTGGTC-3′, *gstf6-1* and right, 5′-GACCCCAAATTTGTAATTGTACCAG-3′) and LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′).

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. The primer sequences for RT-PCR were 5′-GGTTGTGAAACTATATGGACAGG-3′ and 5′-TCAGTGACCAGCCAGCACC-3′ for *TT19*, and 5′-GAGTATTCAAGCTTGGTGGCG-3′ and 5′-CAAGACTCATTATCGAAGATTACATT-3′ for *AtGSTF6*. The PCR program comprised an initial denaturation step of 94°C for 3 min; 24 cycles of 94°C for 30 s, 55°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.

For flavonoid analysis, the rosette leaves of the 3-week-old plants were harvested and extracted with 5 μl extraction solvent (methanol : glacial acetic : water = 45 : 5 : 50) per milligram fresh weight of tissue. After centrifugation at 12,000×*g*, the cell debris was discarded, and the extract was centrifuged again. 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.

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 (*TT19*: 5′-AAAAAGCAGGCTCAATGGTTGTGAAACTATATGGACAGG-3′ and 5′- AGAAAGCTGGGTTCAGTGACCAGCCAGCACCATAA-3′; *AtGSTF5*: 5′- AAAAAGCAGGCTGGATGGGCATAAACGCGAGC-3′ and 5′-AGAAAGCTGGGTTTAATTCTTCTTCTTATGGTACCAAGCC-3′). 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. The *attB* site was introduced by 2 steps of PCR using gene-specific primers (for *TT19* and *AtGSTF5*, we used the above mentioned primers; *AtGSTF6*: 5′-AAAAAGCAGGCTCAATGGCAGGAATCAAAGTTTTCGG-3′ and 5′-AGAAAGCTGGGTTTAAAGAACCTTCTGAGCAGAAGGC-3′) and *attB* adaptor primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′). Entry clones were then obtained by BP recombination 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. Recombinant proteins with a 6-histidine (6×His) tag at the N-terminal extension were expressed in *E. coli* BL21-AI™. 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 ml^{-1} 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, Marvland, 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.

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.

Phylogenetic analysis of the deduced amino acid sequences of *Arabidopsis* GSTs and known GSTs that are involved in anthocyanin biosynthesis were performed using the neighbor-joining method (Figure 1). The phylogenetic tree showed that TT19, AtGSTF5, and AtGSTF6 were classified in the phi class, as were AN9 from petunia (*Petunia hybrida*) and PfGST1 from perilla (*Perilla frutescens*); in contrast, Bz2 from maize (*Zea mays*) was classified into the tau class.

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*,

Table 1. GST activity of purified TT19, AtGSTF5 and AtGSTF6 recombinant proteins against CDNB

Proteins	GST specific activity (nmol min^{-1} μg^{-1} protein)
Standard GST from rat liver	383.1 ± 0.1
TT19	0.52 ± 0.02
AtGSTF5	0.36 ± 0.02
AtGSTF6	0.87 ± 0.05

Values are means±SD (n=3)

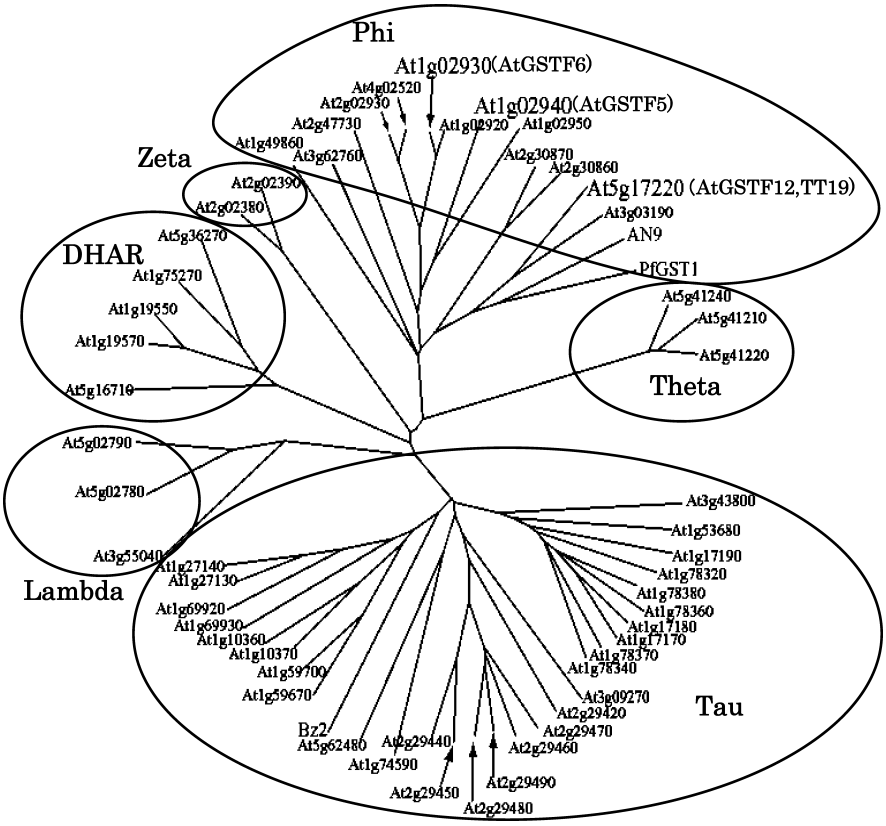

Figure 1. Phylogenetic analysis of *Arabidopsis* GST homologues. Phylogenetic analysis of *Arabidopsis* GST-like proteins using amino acid sequences from GenBank database presented in the *Arabidopsis* genome initiative (AGI) code. Phylogenetic tree was constructed using the ClustalW program with the 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.

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).

The T-DNA-inserted mutants of *TT19* (Salk_105779, *gstf12-1*) and *AtGSTF6* (Salk_026398, *gstf6-1)* were analyzed in terms of relevant gene expression and flavonoid accumulation. The *gstf12-1* line contained a T-DNA insertion at the second intron of At5g17220 and the *gstf6-1* line, at the first intron of At1g02930 (Figure 2A). The expressions of *TT19* and *AtGSTF6* were analyzed by RT-PCR. 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 2B). As shown in Figure 3B, 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 3A, B). These results suggest that *TT19* is almost exclusively involved in anthocyanin accumulation; the mutation in *TT19* thus

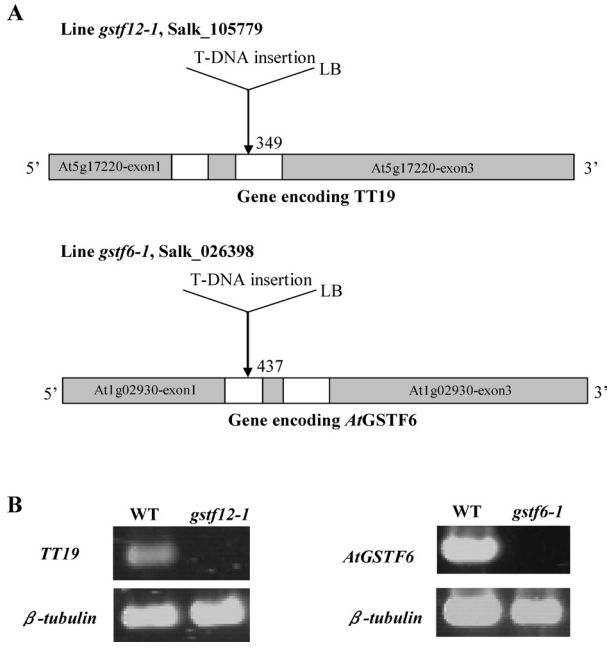

Figure 2. T-DNA-inserted mutants of *TT19* (At5g17220) and *AtGSTF6* (At1g02930). A. Schematic structure of the T-DNA-inserted lines of *TT19* (At5g17220) (line *gstf12-1*, Salk_105779) and *AtGSTF6* (At1g02930) (line *gstf6-1*, Salk_026398). B. Expression of the *TT19* and *AtGSTF6* genes in mutant 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.

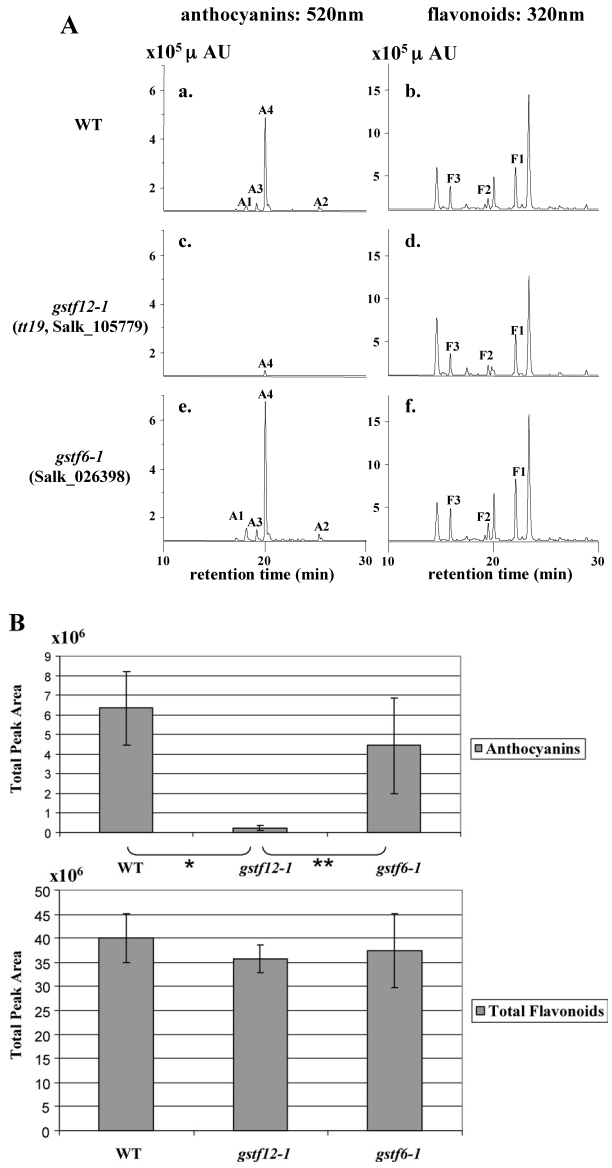

Figure 3. Flavonoid accumulation in knockout mutants. A. HPLC/PDA chromatograms of rosette leaf extract from wild-type (WT) *Arabidopsis*, the *tt19* knockout mutant (*gstf12-1*), and the *AtGSTF6* knockout mutant (*gstf6-1*). (a, c, e) Absorbance at 520 nm for detection of anthocyanins. (b, d, f) Absorbance at 320 nm for detection of flavonoids. 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.

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 (*; $p<0.05$, **; $p<0.005$).

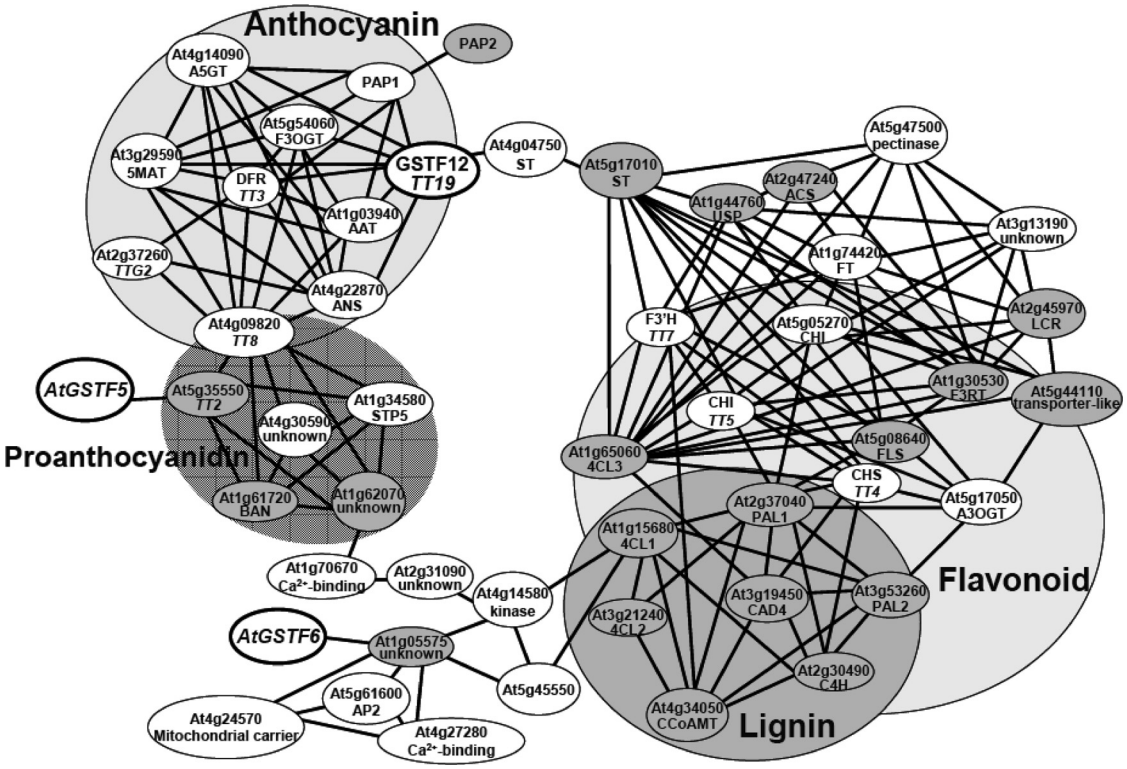

Figure 4. Co-expression networks of *PAP1*-upregulated genes with whole genes. Co-expression analysis was performed using the "Correlated Gene" program available at RIKEN PRIMe (http://prime.psc.riken.jp/) based on ATTED-II, and the results were drawn using the Pajek program. The genes up-regulated by *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.

causes a change in the anthocyanin accumulation level, and no other genes including *AtGSTF6* and possibly *AtGSTF5*, can complement.

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, which allows co-expression analyses based on publicly available microarray data via a co-expression 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 4, 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.

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