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Arabidopsis TT19 Functions as a Carrier to Transport Anthocyanin from the Cytosol to Tonoplasts

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ABSTRACT Anthocyanins are synthesized in the cytosolic surface of the endoplasmic reticulum (ER) but dominantly accumulate in the vacuole. Little is known about how anthocyanins are transported from the ER to the vacuole. Here, we provide evidence supporting that Transparent Testa 19 (TT19), a glutathione *S*-transferase (GST), functions as a carrier to transport cyanidin and/or anthocyanins to the tonoplast. We identified a novel *tt19* mutant (*tt19-7*), which barely accumulates anthocyanins but produces a 36% higher level of flavonol than the wild-type (WT), from ethyl methanesulfonate mutagenized seeds. Expressing TT19-fused green fluorescence protein (GFP) in *tt19-7* rescues the mutant phenotype in defective anthocyanin biosynthesis, indicating that TT19–GFP is functional. We further showed that TT19–GFP is localized not only in the cytoplasm and nuclei, but also on the tonoplast. The membrane localization of TT19–GFP was further ascertained by immunoblot analysis. *In vitro* assay showed that the purified recombinant TT19 increases water solubility of cyanidin (Cya) and cyanidin-3-*O*-glycoside (C3G). Compared with C3G, Cya can dramatically quench the intrinsic tryptophan fluorescence of TT19 to much lower levels, indicating a higher affinity of TT19 to Cya than to C3G. Isothermal titration calorimetry analysis also confirmed physical interaction between TT19 and C3G. Taken together, our data reveal molecular mechanism underlying TT19-mediated anthocyanin transportation.

Key words: anthocyanin; cyanidin; transportation; TT19; *Arabidopsis*.

INTRODUCTION

In higher plants, anthocyanins are a class of water-soluble flavonoid pigments having orange red to violet and blue, depending on the vacuolar pH and various modifications of the core. They are usually accumulated in leaves, stems, flowers, fruits, and seed coats, etc., in response to environmental and developmental cues (Jackson et al., 1992; Chalker-Scott, 1999; Steyn et al., 2002). Anthocyanins can function as visual signals to attract pollinators and seed dispersers, as antioxidants to scavenge reactive oxygen species, and as filters to absorb UV and high light irradiation (Christie et al., 1994; Winkel-Shirley, 2001; Lorenc-Kukula et al., 2005; Castellarin et al., 2007). Thus, anthocyanins are suggested to play an important role in plant defenses to various abiotic and biotic stresses and interactions between plants and insects or microbes. The fascinating phenotypes, such as color streak and variegation, caused by deficiency in anthocyanin biosynthesis have long been excellent genetic markers to address a number of basic biological questions, leading to discovery of milestone concepts such as transposable elements, gene silencing (co-suppression), and paramutation. Anthocyanins have also been an important agronomic trait for genetic modification not only to beautify our living environment, but also to improve food quality and human health (Lila, 2004; Butelli et al., 2008; He and Giusti, 2010). Anthocyanin biosynthesis therefore is intensively studied in many plant species including crops and garden plants.

Anthocyanins are synthesized through a conserved branch of well-characterized flavonoid biosynthetic pathways on the cytoplasmic surface of the endoplasmic reticulum (ER). The enzymes involved in the pathway loosely form a multi-enzyme complex, viz. metabolon, in which the membrane-spanning cytochrome P450 enzymes including cinnamate 4-hydroxylase (C4H), flavanone-3β-hydroxylase (F3H), and flavonoid 3′-hydroxylase (F3′H) function as scaffolds to assemble the

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soluble subunits on the ER (Winkel-Shirley, 1999; Jørgensen et al., 2005). Expression of the polypeptide components required for metabolon formation is coordinately regulated by a ternary complex composed of the basic helix-loop-helix (bHLH) and R2R3-MYB transcriptional factors, and WD40 cofactors (Koes et al., 2005; Hichri et al., 2011). It seems that plants have evolved an efficient system to rapidly produce anthocyanins whenever necessary. In contrast to the biosynthetic pathway, the process of anthocyanin transport from the ER biosynthetic site to the final destination vacuole is poorly understood (Winkel-Shirley, 2001; Zhao and Dixon, 2010). In general, two models have been proposed for anthocyanin import into vacuoles: the vesicle trafficking-mediated model and the transporter-mediated model (Grotewold and Davis, 2008; Zhao and Dixon, 2010). The former is established based on the observation that anthocyanins are accumulated first in vesicle-like structures, also called anthocyanoplasts, and then imported into the central vacuole probably in a vesicle fusion manner (Grotewold et al., 1998; Pourcel et al., 2010; Gomez et al., 2011). However, it remains unknown whether proteins involved in the vesicle trafficking machinery affect the process of anthocyanin sequestration in the vacuole. The latter is strongly supported by genetic and biochemical evidence that the vacuole-localized multidrug and toxic compound extrusion (MATE) transporter, such as the TT12, acts as a cyanidin-3-*O*-glucoside/H^+-antiporter (Debeaujon et al., 2001; Marinova et al., 2007); and its ortholog MATE1 from *M. truncatula* preferentially transport epicatechin 3′-*O*-glucoside, the proposed precursor for the synthesis of proanthocyanidins to the vacuole (Zhao and Dixon, 2009). In addition, the MRP-type ATP binding cassette (ABC) transporter has also been implicated in anthocyanin transport into the vacuole (Goodman et al., 2004). Interestingly, in both vesicle trafficking and transporter-mediated systems, a glutathione *S*-transferase (GST) is proposed to assist in efficient transport of anthocyanins from the ER to the vacuole (Marrs et al., 1995; Mueller et al., 2000; Kitamura et al., 2004; Gomez et al., 2011).

The important role of a GST in anthocyanin biosynthesis was first demonstrated in maize by its mutant *bronze-2* (*bz2*) defective in anthocyanin accumulation and its biochemical activity in conjugating cyanidin-3-*O*-glucoside (C3G) with glutathione (Marrs et al., 1995). The anthocyanin–glutathione conjugate was thought to be recognized by a tonoplast-localized pump, and subsequently transported into vacuoles (Marrs et al., 1995; Alfenito et al., 1998). Homologs of the *Bz2* gene have been identified in a number of plant species including *Arabidopsis* (*TT19*), petuna (*AN9*), carnation (*Fl3*), and grape (*VvGST1* and *VvGST4*), indicating the highly conserved function of GSTs in anthocyanin biosynthesis (Alfenito et al., 1998; Mueller et al., 2000; Larsen et al., 2003; Kitamura et al., 2004; Conn et al., 2008). To date, however, evidence is increasing against the suggestion that GSTs function as an enzyme in anthocyanin biosynthesis. For example, glutathioned anthocyanin molecules have not been found in plant cells; the petunia glutathione *S*-transferase AN9 is unable to catalyze conjugation of glutathione to anthocyanins or other flavonoids *in vitro* (Mueller et al., 2000); recently identified tonoplast-localized transporters prefer to transport the acylated flavonoids to the vacuole (Gomez et al., 2009; Zhao et al., 2011). Thus, it was proposed that GSTs may act as a carrier or ligandin to escort anthocyanins on the way from the ER to the tonoplast (Kitamura, 2006; Zhao and Dixon, 2010). Although their interaction was inferred from results of some assays such as equilibrium dialysis and tryptophan quenching with the recombinant GSTs (Mueller et al., 2000), direct evidence for GSTs to bind anthocyanins is still lacking. Here, we show that *Arabidopsis* TT19 is localized both in the cytoplasm and on the tonoplast, and it can directly bind to cyanidin and C3G but not conjugate them with glutathione, supporting the hypothesis that TT19 is a carrier protein for sequestering anthocyanins into the vacuole.

RESULTS

Identification of a New Mutant of *TT19*

We screened about 20 g of ethyl methanesulfonate mutagenized seeds of *Arabidopsis* for the reduced accumulation of anthocyanins in leaves. In this study, we characterized one mutant with no visible anthocyanins accumulated in cotyledons and hypocotyls of seedlings grown on the half MS medium supplemented with 5% of sucrose, hereafter referred to as the anthocyanin induction media (AIM) (Figure 1A). The mutant also displays yellowish seed coats at the ripening stage, but the seed color can be gradually turned into brown and eventually reached the same color as the wild-type (WT) (data not shown). These phenotypes of the mutant are very similar to those reported in *tt19* mutants (Kitamura et al., 2004). To clone the gene, an F_2 mapping population was generated by crossing the mutant with Landsberg *erecta*. Using simple sequence length polymorphism (SSLP) markers, we mapped the locus to the region between markers nga106 and mpi7 (Figure 1B). This region contains the gene *TT19* (At5g17220), which is composed of three exons and two introns (Figure 1C). DNA sequencing revealed a transition of G to A at the first nucleotide of the second intron in *tt19-7* (Figure 1C). To determine whether this point mutation affects intron splicing of the *TT19* transcripts, we amplified the open reading frame (ORF) of *TT19* by RT–PCR. Two bands were detected in *tt19-7* while only one was detected in WT (Figure 1D). Sequencing these fragments showed that the larger fragment (795 bp) contained two introns whereas the smaller one (596 bp) lacked the exon 2 compared with the WT fragment (Figure 1D), indicating that the larger fragment in the mutant resulted from the absence of splicing, whereas the smaller one resulted from abnormal splicing. Both the larger and smaller mRNAs transcribed in the mutant are predicted to generate a premature stop codon. Thus, we conclude that the new allele of *tt19* is a null mutant, and designate it as *tt19-7*, since six alleles of *tt19* have been identified (Kitamura et al., 2004; Hsieh and Huang 2007; Li et al., 2011).