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Molecular and biochemical characterization of the UDP-glucose: Anthocyanin 5-*O*-glucosyltransferase from *Vitis amurensis*

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ABSTRACT

Generally, red *Vitis vinifera* grapes only contain monoglucosidic anthocyanins, whereas most non-*vinifera* red grapes of the *Vitis* genus have both monoglucosidic and bis-glucosidic anthocyanins, the latter of which are believed to be more hydrophilic and more stable. Although previous studies have established the biosynthetic mechanism for formation of monoglucosidic anthocyanins, less attention has been paid to that of bis-glucosidic anthocyanins. In the present research, the full-length cDNA of UDP-glucose: anthocyanin 5-*O*-glucosyltransferase from *Vitis amurensis* Rupr. cv. 'Zuoshanyi' grape (Va5GT) was cloned. After acquisition and purification of recombinant Va5GT, its enzymatic parameters were systematically analyzed *in vitro*. Recombinant Va5GT used malvidin-3-*O*-glucoside as its optimum glycosidic acceptor when UDP-glucose was used as the glycosidic donor. Va5GT-GFP was found to be located in the cytoplasm by analyzing its subcellular localization with a laser-scanning confocal fluorescence microscope, and this result was coincident with its metabolic function of modifying anthocyanins in grape cells. Furthermore, the relationship between the transcriptional expression of *Va5GT* and the accumulation of anthocyanidin bis-glucosides during berry development suggested that Va5GT is a key enzyme in the biosynthesis of bis-glucosidic anthocyanins in *V. amurensis* grape berries.

1. Introduction

Accumulated widely in almost all plant organs, including roots, stems, leaves, flowers, berries and seeds, anthocyanins are a group of water-soluble flavonoid pigments synthesized from phenylalanine-derived plant secondary metabolites (Mazza, 1995; Winkel-Shirley, 2001). Normally, in pink, red, purple and black grapes, the accumulation of anthocyanins in berry skins determines the color of the grapes. The anthocyanin composition and content of red grapes are affected by various intrinsic factors, such as species and varieties, and many external factors, which results in noticeable differences between different grape varieties (Downey et al., 2006). Therefore, in the past several decades, much research has focused on anthocyanin biosynthesis in grapes.

The biosynthetic pathways to anthocyanins in various plants have been presented in detail in previous reports (Heller and Forkmann, 1993). Anthocyanins are first visible in grapes when the berries begin to expand at the start of veraison, and this occurs simultaneously with the rapid accumulation of sugar (Hrazdina et al., 1984). Anthocyanins are formed by a sequence of metabolic steps that can be divided into two different stages of reactions: production of anthocyanidin-3-*O*-glucosides (**2**, **4**, **6**, **8**, **10**, **12**) in Fig. 1, and further modifications of those glucosides (Martin et al., 1993). Generally, the glycosylation of an anthocyanidin or anthocyanin is catalyzed by uridine diphosphate glycosyltransferases (UGTs), which are characterized by a signature motif, the conserved plant secondary product glycosyltransferase box (PSPG) (Hughes and Hughes, 1994). This sugar conjugation modifies the anthocyanin, giving it increased water solubility and chemical stability, which could also facilitate the transfer of anthocyanins from their cytoplasmic production site into vacuoles, where anthocyanins finally accumulate (Nakatsuka et al., 2008). It is believed that anthocyanidins are first glycosylated at the 3-*O*-position by UDP-glucose: flavonoid 3-*O*-glucosyltransferase (3GT) to form their corresponding monoglucosidic anthocyanins. Then, the bis-glucosidic anthocyanins can be synthesized by the action of another member of the UGT family, UDP-glucose: anthocyanin 5-*O*-glucosyltransferase (5GT). To date, the genes encoding 3GTs

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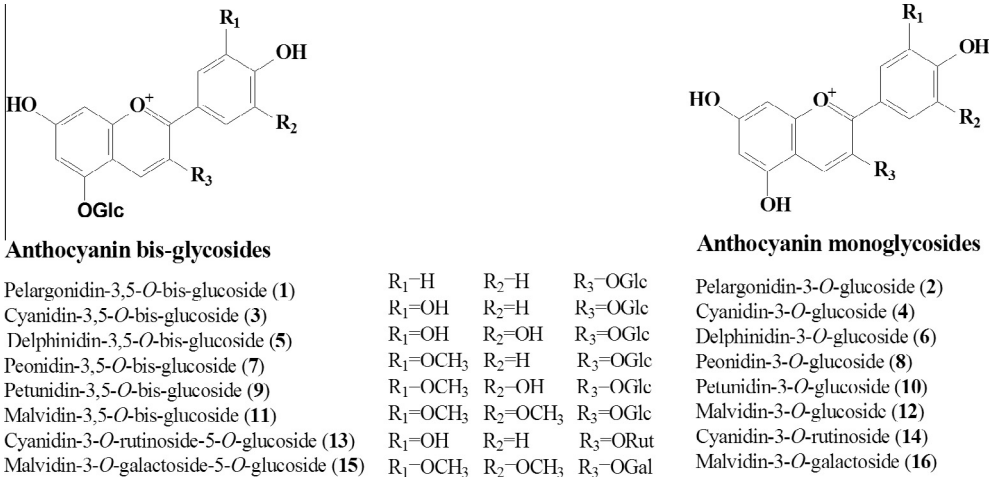

Fig. 1. Chemical structure of anthocyanidin monoglycosides (**2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**) and their corresponding bis-glycosides (**1**, **3**, **5**, **7**, **9**, **11**, **13**, **15**). Glc, Rut and Gal are abbreviations for glucoside, rutinoside and galactoside, respectively.

have been isolated and well characterized in several different grape species and varieties (Ford et al., 1998; Hall et al., 2012; Offen et al., 2006; Sparvoli et al., 1994). However, only a few studies have reported the isolation of *5GT* genes or their enzymes from a limited number of grape germplasms (Jánváry et al., 2009; Neumann et al., 2006). Jánváry et al. (2009) cloned two *5GT* alleles (functional *Cha5GT* and nonfunctional *Dia5GT*) from the heterozygous hybrid cultivar 'Regent', which is a cross of *V. vinifera* cv. 'Diana' and the interspecific hybrid cv. 'Chambourcin'. A functional analysis of Cha5GT and Dia5GT established that two mutations in the *5GT* gene disrupt its enzymatic activity. Because of the absence of active 5GT in *V. vinifera* red grapes, they only accumulate anthocyanidin monoglucosides (**2**, **4**, **6**, **8**, **10**, **12**), but not their corresponding bis-glucosides (**1**, **3**, **5**, **7**, **9**, **11**), which are widely found in the mature red grapes of almost all non-*vinifera* species (Mazzuca et al., 2005).

In recent years, *V. amurensis* grapes have been the focus of several studies, particularly with regards to their ability to produce resveratrol and their cold hardiness and fungal disease resistance (Kiselev et al., 2007; Kovács et al., 2003; Wan et al., 2007). Unlike *V. vinifera* grapes, the berry skins of *V. amurensis* species usually accumulate abundant anthocyanidin-3,5-*O*-bis-glycosides (**1**, **3**, **5**, **7**, **9**, **11**), due to their highly active 5GT (He et al., 2010a). However, little has been reported on the gene sequences and biochemical functions of 5GT from *V. amurensis* grapes, and no thorough enzymatic characterization of a grape 5GT has been reported previously.

In this study, the full-length cDNA sequence of *Va5GT* from *V. amurensis* Rupr. cv. 'Zuoshanyi' was cloned and used for heterologous expression of the Va5GT protein for its biochemical characterization. A green fluorescent protein (GFP)-tagged Va5GT protein was also localized in *Arabidopsis* leaf cell protoplasts, as determined by laser scanning confocal microscopy. Furthermore, the comparison of *Va5GT* transcriptional expression and the resulting accumulation of bis-glucosidic anthocyanins (**3**, **5**, **7**, **9**, **11**) in grape skins during berry development showed that Va5GT is the crucial structural enzyme in the biosynthesis of bis-glucosidic anthocyanins in these grapes.

2. Results and discussion

2.1. Molecular cloning and bioinformatics analysis of Va5GT

The oligonucleotide primers used in the previous study of *Cha5GT* were selected to amplify *Va5GT* (Jánváry et al., 2009). A nucleotide sequence analysis demonstrated that the full-length *Va5GT* (Genbank accession KF996717) contained an open reading frame of 1395 bp, which was in 99.6% agreement with the previously characterized gene *Cha5GT* (the nucleotide sequence of *Cha5GT* were offered by Prof. Schwab). Thus, *Cha5GT* and *Va5GT* could be considered homologous genes from different grape species. The ExPASy website (http://web.expasy.org/compute_pi/) was used to analyze the encoded amino acid sequence of *Va5GT*, and the Va5GT protein had an estimated isoelectric point of 5.12 and a predicted molecular weight of 51.5 kDa.

An analysis of the Va5GT amino acid sequence using the Pfam 24.0 website (http://pfam.sanger.ac.uk/search/sequence) showed that Va5GT belongs to the GT-B clan of the UGTs family, which contains a diversity of glycosyltransferases (Coutinho et al., 2003). Based on the amino acid sequences of various functionally characterized plant glucosyltransferases that are related to anthocyanin biosynthesis and of the obtained Va5GT, a neighbor-joining phylogenetic tree was constructed. As shown in Fig. 2, three clusters were generally grouped based on *in vitro* regio-selectivity rather than substrate specificity, and Va5GT was placed in cluster II (Vogt and Jones, 2000). In this phylogenetic tree, cluster I exclusively contained flavonoid 3-*O*-glycosyltransferases. Cluster II mainly included anthocyanin 5-*O*-glycosyltransferases and some flavonol 7-*O*-glucosyltransferases. Among them, Va5GT exhibited 40.9–55.5% identities with 5GTs with similar biochemical functions in other plants. The enzymes in cluster III could potentially catalyze the second glycosylation of other anthocyanins or flavonoid glycosides, and usually used a glycosidic donor other than UDP-glucose. For example, the recombinant UDP-xylose: anthocyanidin-3-*O*-galactoside 2″-*O*-xylosyltransferase (AcA3Ga2″XT) from *Actinidia chinensis* catalyzed the production of cyanidin-3-*O*-xylo-galactoside using cyanindin-3-*O*-galactoside and UDP-xylose as substrates (Montefiori et al., 2011).

The alignment of the protein sequences of Va5GT and other glucosyltransferases in grapes showed that Va5GT contained a common C-terminal domain of the UGT superfamily (Fig. S1). Glutamine and histidine residues were highly conserved as the last amino acid residues of the PSPG boxes in glucosyltransferases and galactosyltransferases, respectively. The glutamine residue at the end of the PSPG box in Va5GT suggested that Va5GT might use UDP-glucose as its sugar donor rather than UDP-galactose (Kubo et al., 2004). A previous study confirmed that a truncation at the C-terminus and a V110L transition in Vv5GT disrupted its enzymatic activity in the production of anthocyanin-3,5-*O*-bis-glucosides (Jánváry et al., 2009). Fortunately, the protein sequence