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Genome-wide identification of glycosyltransferases converting phloretin to phloridzin in *Malus* species

Kun Zhou, Lingyu Hu, Pengmin Li, Xiaoqing Gong*, Fengwang Ma*

State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A & F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Phloridzin (phloretin 2′-*O*-glucoside) is the most abundant phenolic compound in *Malus* species, accounting for up to 18% of the dry weight in leaves. Glycosylation of phloretin at the 2′ position is the last and key step in phloridzin biosynthesis. It is catalyzed by a uridine diphosphate (UDP)-glucose:phloretin 2′-*O*-glucosyltransferase (P2′GT), which directly determines the concentration of phloridzin. However, this process is poorly understood. We conducted a large-scale investigation of phloridzin accumulations in leaves from 64 *Malus* species and cultivars. To identify the responsible P2′GT, we performed a genome-wide analysis of the expression patterns of UDP-dependent glycosyltransferase genes (*UGT*s). Two candidates were screened preliminarily in *Malus* spp. cv. Adams (North American Begonia). Results from further qRT-PCR analyses of the genotypes showed a divergence in phloridzin production. Our assays of enzyme activity also suggested that MdUGT88F4 and MdUGT88F1 regulate the conversion of phloretin to phloridzin in *Malus* plants. Finally, when they were silenced in 'GL-3' ('Royal Gala'), the concentrations of phloridzin and phloretin (and trilobatin) were significantly reduced and increased, respectively.

1. Introduction

Dihydrochalcones (DHCs) are phenylpropanoids that are very similar in structure to chalcones, the intermediates in flavonoid formation (Fig. 1). The DHC phloridzin, a mono-glycoside of phloretin, has a glucose moiety attached at the 2′-OH position (Fig. 1). It is the most abundant phenolic compound in the leaves of apple (*Malus domestica*), accounting for 14–18% of the total dry weight for that tissue [1,2]. This makes apple unique in the plant kingdom because, in addition to members of the *Malus* genus, only a few other plants, i.e., *Camellia japonica* [3], *Fragaria* × *ananassa* [4], *Rosa canina* [5], and *Lithocarpus litseifolius* [6], can accumulate tiny amounts of phloridzin [7]. Even *Pyrus* sp., closely related to *M. domestica*, can not accumulate phloridzin and its precursor phloretin [8,9]. Within *Malus*, production of phloridzin varies among cultivars [10,11], developmental stages [12], tissue types [10], and external factors such as pathogen attacks [13]. Phloridzin is enriched in apple seeds, bark, and leaves, but only minimally detected in fruit [10,14].

Phloridzin and its derivatives are excellent antioxidants [15–17], and are widely investigated in the field of human health research [18,19]. Evidence from studies with diabetic mice has shown that phloridzin blocks glucose adsorption in liver and intestinal cells by inhibiting sodium-linked glucose transporters, thereby ameliorating the effects of hyperglycemia [20–22]. However, the benefits of phloridzin in plants are largely unknown, although it is thought to be involved in pathogen resistance. When plants are attacked by a pathogen, phloridzin is hydrolyzed into phloretin by specific cytosolic glucosidases after cellular decompartmentalization. It is then oxidized by peroxidase and/or polyphenol oxidase into *o*-diphenols and toxic *o*-quinoids to inactivate microbial proteins [23,24]. Despite these preliminary reports, the role of phloridzin in pathogen defenses remains unclear because such accumulations have not yet been proven to be relevant to the resistance mechanism [9]. Some researchers have proposed that various metabolic pathways of DHCs, occurring in different apple genotypes, determine the potential for resistance to fire blight [25]. Petkovšek et al. have suggested that a consistent level of polyphenols is not necessary in the plant, but that their post-infection accumulation and further conversion are prerequisites for making plants more resistant to such threats [26]. Overexpression of *chalcone 3-hydroxylase* (CH3H) from *Cosmos sulphureus* stimulates the accumulation of 3-hydroxyphlorizin and reduces the susceptibility of *M. domestica* cv. Pinova to fire blight and scab [27]. Phloretin also displays broad-spectrum

Abbreviations: DHC, dihydrochalcone; GTs, glycosyltransferases; HPLC, high performance liquid chromatography; PSPG, plant secondary product glycosyltransferase; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse-transcription PCR; UDP, uridine diphosphate; UGTs, UDP-dependent glycosyltransferases

* Corresponding authors.
E-mail addresses: gongxq0103@nwsuaf.edu.cn (X. Gong), fwm64@sina.com, fwm64@nwsuaf.edu.cn (F. Ma).

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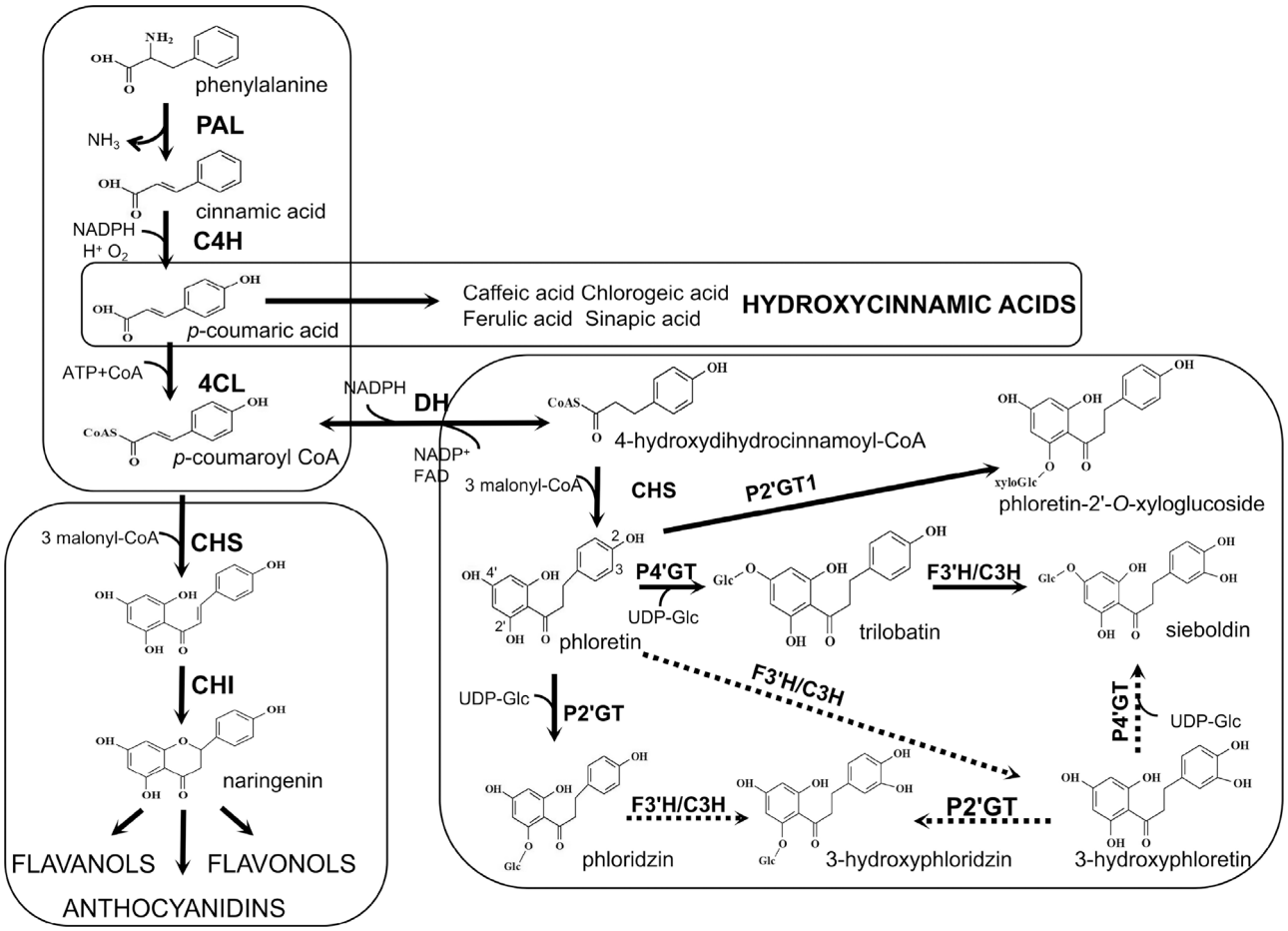

Fig. 1. Biosynthetic pathway of dihydrochalcones. PAL, phenylalanine ammonia lyase;.
C4H, cinnamate-4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; DH, dehydrogenase; P2′GT, UDP-glucose:phloretin 2′-*O*-glycosyltransferase; P2′GT1, UDP-xyloglucose:phloretin 2′-*O*-glycosyltransferase; P4′GT, UDP-glucose:phloretin 4′-*O*-glycosyltransferase; F3′H, flavonoid 3′-hydroxylase; C3H, chalcone-3-hydroxylase. Dotted arrow represents proposed pathway.

antibacterial activity and much stronger antioxidant activity [16,28], and is considered as an active compound against fungal infections [26].

Glycosylation, mediated by glycosyltransferases (GTs; EC 2.4.x.y), is ubiquitous in all living organisms [29]. According to CAZy (http://www.cazy.org/GlycosylTransferases.html), the GTs from various species can be classified into 94 families. The largest, Family 1, comprises diverse enzymes from animals, plants, bacteria, fungi, and viruses. In plants, Family-1 GTs, also called uridine diphosphate (UDP)-dependent glycosyltransferases, or UGTs, utilize UDP-activated sugars as the major donor molecule. One UGT feature is a conserved UGT-defining motif close to the C-terminus. The plant secondary product glycosyltransferase (PSPG) motif has 44 amino acid residues at the UDP-sugar binding site. A wide array of small molecules, including hormones, lipophilic acceptors, and secondary metabolites, can be glycosylated by UGTs, which improves their solubility in water and their chemical stability, reduces their toxicity, and changes their biological activity in plants [30,31]. Phloridzin is synthesized in three steps (Fig. 1), the final one being the glycosylation of phloretin [1,32,33]. Because this glycosylation has rarely been reported, it is essential that we identify the UDP-glucose:phloretin 2′-*O*-glucosyltransferase (P2′GT) in *Malus* plants if we are to conduct a more thorough investigation of the physiological relevance of phloridzin. To date, only four candidates have been isolated from apple leaves and tested for substrate specificity in vitro [1,32–34].

Apple is one of the most important crop trees because its popularly consumed fruit are available year-round in markets. Because those plants contain high levels of beneficial phloridzin, more research is being focused on elucidating its mechanism of biosynthesis and its physiological relevance. In this study, we sampled the leaves of 64 *Malus* species and cultivars grown in the same location and measured their amounts of DHCs (i.e., phloretin, phloridzin, trilobatin, and sieboldin). Among them, eight genotypes exhibited a novel mode of phloridzin metabolism. Each of the tested samples featured a different phloridzin profile, which we used to determine whether phloridzin levels are correlated with the expression of *UGT*s across the entire genome. Protein activity as well as the transgenic plants analysis demonstrated that both MdUGT88F1 and its paralog MdUGT88F4 convert phloretin to phloridzin in apple. These findings will be beneficial to future examinations of the physiological role and mechanism of phloridzin biosynthesis.

2. Materials and methods

2.1. Plant materials, growing conditions, treatments, and chemicals

This study involved 64 *Malus* species and cultivars (Supplemental Table 1) plus one other cultivar, ‘Royal Gala’. For the quantification analysis of DHCs, we collected mature leaves, branches, bark, and open flowers from healthy trees growing at the Horticultural Experimental Station and on the campus of Northwest A & F University, Yangling (34°20 N, 108°24 E), China. For each tissue type, at least five samples were harvested from individual trees at 9:00 AM, then immediately frozen in liquid nitrogen and stored at −80 °C.

The sources for chemicals were Yuanye (Shanghai, China) for